AN ABSTRACT OF THE THESIS OF

Justin A. Schulze for the degree of Master of Science in Horticulture presented on February 10, 2017.

Title: Breeding, Production, and Morphophysiology Among Cytotypes of Common and Portuguese Cherrylaurel.

Abstract approved:

_____________________________________________________________________

Ryan N. Contreras

The common cherrylaurel (*Prunus laurocerasus*) and Portuguese cherrylaurel (*P. lusitanica*) are economically important landscape shrubs across the United States. To gain a better understanding of physiology and morphology in these higher level polyploids (*P. lusitanica, 2n = 8x; P. laurocerasus, 2n =22x*), a variety of breeding experiments were performed. The long-term goal of these experiments was to lay the ground-work for the creation of a sterile, shothole disease resistant cherrylaurel, while maintaining its desirable ornamental features.

In attempt to narrow the gap in ploidy level between these two species, the chromosome number of *P. lusitanica* was doubled using colchicine and oryzalin, and differences in cytotypes were observed. This was performed with the hope of performing interspecific hybridization and introgression of shothole disease resistance in to *P. laurocerasus*. The most effective method was found to be oryzalin at a rate
of 125 µM, and induced polyploids were shorter, had thicker leaves, and larger but fewer stomata.

Preliminary research was performed to germinate immature *P. lusitanica* seed, in vitro, to develop a putative embryo rescue medium for use in this interspecific hybridization. The most significant factor in the media for successful germination was sucrose, with 30 g·L⁻¹ of sucrose media germinating significantly more seed than 60 g·L⁻¹. Also, germination only occurred at 12 weeks after pollination, with no germination at 6 and 9 weeks regardless of cold-stratification.

Existing chromosome doubled cytotypes of *P. laurocerasus* were compared to the standard ploidy cytotype. Differences in vegetative propagation were observed and reported in the Schipkaensis cultivar. Chromosome doubled *P. laurocerasus* ‘Schipkaensis’ rooted at an equivalent percentage, had equivalently long roots, and had fewer roots per cutting. Deceased number of roots produced no apparent detrimental effects in propagation success.

In cytotypes of the Otto Luyken cultivar, numerous morphological and physiological were made. Chromosome double forms were generally shorter, smaller in width and conical volume, and had larger but fewer stomata. The also had increased stomatal conductance and transpiration, with decreased water-use efficiency. Size and biomass observations were used to estimate carbon fixation, and chromosome doubled Otto Luyken cultivars were estimated to fix significantly less carbon.
Master of Science thesis of Justin A. Schulze presented on February 10, 2017

APPROVED:

Major Professor, representing Horticulture

Head of the Department of Horticulture

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Justin A. Schulze, Author
I would like to express my sincere gratitude to all the awesome people who made the completion of this work possible. In no particular order, I would specifically like to thank;

Dr. Ryan Contreras: You took a chance on me as a student with zero formal research or horticultural training. I truly appreciate the opportunity and the vast experience I have received as part of my education.

Jason Lattier: As a veteran of the graduate school experience there were few who I relied on more for navigation and advice in my studies. Thank you brother.

Kim Shearer-Lattier: You were there from day one as a welcoming and inclusive presence for the entirety of my graduate experience. You were always up for spirited discussions that expanded my understanding of horticulture.

Hsuan Chen: Your enthusiasm for science, and life in general, is inspiring. I attempted, with varying degrees of success, to mirror your positivity and optimism in my life and work.

Dr. Carolyn Scagel: You walked me through my horrifying, first-round of edits. You calmly reassured me in my panic and I truly appreciate all the time and resources you and your lab have devoted to this work.

Dr. John Lambrinos: You were always willing to let me fire a few questions at you whether in the hallway or just passing by your office. Your laid-back demeanor made every step of this process more manageable.

My Parents, Holly and Paul Schulze: You have always supported all my ventures, regardless of feasibility and practicality. All my accomplishments are a direct result of your unwavering encouragement. Thank you.

My Wife, Elizabeth Schulze: You tolerated my erratic mood swings corresponding to stages of the graduate school process, which closely resembled the five stages of grief. Thank you for your unrelenting emotional support.

My Children, Hazel and August Schulze: Everything I do is for you.
CONTRIBUTION OF AUTHORS

Ryan N. Contreras assisted, advised, and edited in all aspects of every chapter contained herein. Jason D. Lattier performed the initial experiment to develop the optimum tissue culture medium, and advised and edited the remainder of the experiment in Chapter 3. Carolyn F. Scagel advised and edited all chapters herein in addition to providing the means for tissue analysis of Chapter 5. John G. Lambrinos advised and edited on all chapters contained herein.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th></th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>General Introduction</td>
</tr>
<tr>
<td></td>
<td>1.1 Literature Cited</td>
</tr>
<tr>
<td>2</td>
<td>In Vivo Chromosome Doubling of <em>Prunus lusitanica</em> and Preliminary Morphological Observations</td>
</tr>
<tr>
<td></td>
<td>2.1 Chapter Introduction</td>
</tr>
<tr>
<td></td>
<td>2.2 Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>2.3 Results and Discussion</td>
</tr>
<tr>
<td></td>
<td>2.4 Literature Cited</td>
</tr>
<tr>
<td>3</td>
<td>In Vitro Germination of Immature <em>Prunus lusitanica</em> Seed</td>
</tr>
<tr>
<td></td>
<td>3.1 Chapter Introduction</td>
</tr>
<tr>
<td></td>
<td>3.2 Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>3.3 Results and Discussion</td>
</tr>
<tr>
<td></td>
<td>3.4 Literature Cited</td>
</tr>
<tr>
<td>4</td>
<td>Comparative Vegetative Propagation in Two ‘Schipkaensis’ Common Cherry Laurel Ploidy Levels</td>
</tr>
<tr>
<td></td>
<td>4.1 Chapter Introduction</td>
</tr>
<tr>
<td></td>
<td>4.2 Materials and Methods</td>
</tr>
<tr>
<td></td>
<td>4.3 Results and Discussion</td>
</tr>
<tr>
<td></td>
<td>4.4 Literature Cited</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>5.1</td>
</tr>
<tr>
<td>5.2</td>
</tr>
<tr>
<td>5.3</td>
</tr>
<tr>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>6.1</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>8.1</td>
</tr>
<tr>
<td>8.2</td>
</tr>
<tr>
<td>8.3</td>
</tr>
<tr>
<td>8.4</td>
</tr>
<tr>
<td>8.5</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Flow cytometric output of <em>Prunus lusitanica</em> and induced polyploids. Over 6000 particles were run with 2n = 8x, 2n= 12x and 2n =16x, plus the <em>Pisum sativum</em> ‘Ctirad’ internal standard (A)..........................27</td>
<td></td>
</tr>
<tr>
<td>2.2 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) <em>Prunus lusitanica</em>, observed at ×200 magnification.................................28</td>
<td></td>
</tr>
<tr>
<td>2.3 Morphological variation within and between 1.5-year-old 2n = 16x (A) and 2n = 8x (B) <em>Prunus lusitanica</em>............................................................29</td>
<td></td>
</tr>
<tr>
<td>2.4 Stomatal variation between 2n = 16x (A) and 2n = 8x (B) <em>Prunus lusitanica</em>, observed at ×200 magnification..............................................30</td>
<td></td>
</tr>
<tr>
<td>3.1 Different outcomes of in vitro germination of cold-stratified and direct sown <em>Prunus lusitanica</em> collected 6, 9, and 12 weeks after pollination.........................47</td>
<td></td>
</tr>
<tr>
<td>4.1 Adventitious rooting (%), average root length, root number, and total root length on stem cuttings from 22x and 44x ‘Schipkaensis’ common cherrylaurel........61</td>
<td></td>
</tr>
<tr>
<td>4.2 Rooted stem cuttings from 22x and 44x ploidy levels of ‘Schipkaensis’ common cherrylaurel..........................................................62</td>
<td></td>
</tr>
<tr>
<td>5.1 Width, height, (A) and volume (B) measurements comparing three independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’.................................85</td>
<td></td>
</tr>
<tr>
<td>5.2 Photographs of plants before the T1 (December 2015) destructive harvest.........86</td>
<td></td>
</tr>
<tr>
<td>5.3 Material breakdown of two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’...............................................................88</td>
<td></td>
</tr>
<tr>
<td>5.4 Linear regression and best-fit line of total biomass by conical volume using two <em>Prunus laurocerasus</em> ‘Otto Luyken’ cytotypes grown in 26.5-liter containers.....88</td>
<td></td>
</tr>
<tr>
<td>5.5 Estimated annual carbon fixation of two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’. .................................................................89</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>5.6 Stomatal comparison between two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’</td>
<td>90</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Sources and quantities of altered-ploidy <em>Prunus lusitanica</em> seedlings</td>
<td>23</td>
</tr>
<tr>
<td>2.2 Stomatal density and guard cell length in three cytotypes of one-year-old <em>Prunus lusitanica</em></td>
<td>24</td>
</tr>
<tr>
<td>2.3 Morphological observations of three cytotypes of 1.5-year-old <em>Prunus lusitanica</em></td>
<td>25</td>
</tr>
<tr>
<td>2.4 Leaf color values in three cytotypes of 1.5-year-old <em>Prunus lusitanica</em> collected using a colorimeter (BC-10, Konica Minolta, Ramsey, NJ)</td>
<td>26</td>
</tr>
<tr>
<td>3.1 Radicle emergence, shoot emergence and shoot length of immature (12 weeks after pollination) <em>Prunus lusitanica</em> seeds without testa in experiment I</td>
<td>45</td>
</tr>
<tr>
<td>3.2 Radicle emergence and shoots emergence of immature <em>Prunus lusitanica</em> seeds with testa cultured at three different collection times in experiment II</td>
<td>46</td>
</tr>
<tr>
<td>5.1 Least squared (LS) means ± LS standard error of weighted whole-plant measurements comparing two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’</td>
<td>83</td>
</tr>
<tr>
<td>5.2 Stomatal and portable photosynthesis system (LI-6400XT; LI-COR Inc., Lincoln, NE) measurements comparing two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of <em>Prunus laurocerasus</em> ‘Otto Luyken’</td>
<td>84</td>
</tr>
</tbody>
</table>
DEDICATION

I would like to dedicate this work to my daughter Hazel. The day I found out your mom was pregnant I bought a graduate record exam study book. You inspired me to pursue a graduate education that I had continued to put off. By the time you were one, I was accepted to Oregon State University for a M.S. in Horticulture.

Raising you and your brother has made me a more patient and resilient person. I love you kid, and I owe it all to you.
General Introduction

Rosaceae is an economically important plant family which is highly diverse in the northern hemisphere (Hummer and Janick 2009; Judd, 1999). This family takes many forms, from herbs to trees, typically with alternate leaf arrangement and simple or compound leaf forms. Rosaceae contains many important fruit crops such as apples, pears, raspberries, strawberries, and many stone fruits (Janick, 2005). It also contains many important ornamental genera such as *Cotoneaster, Crataegus, Malus, Photinia, Prunus,* and *Rosa.* Flowers are often described as showy which provides insight to the many rosaceous ornamental cultivars.

Ornamental *Prunus* are often associated with the dramatic Japanese flowering cherry (*P. serrulata*) which is widely planted with numerous cultivars. However, there is a broad range of ornamental *Prunus* species, from a variety of cultivated apricots, cherries, peaches, plums, and cherrylaurels (Dirr, 2009). This genus, often referred to simply as stone fruits, produces drupes containing a stony endocarp, and can be either deciduous or evergreen with simple, often serrate leaf structure. Within this genus, cherrylaurels are economically important species that are often overlooked.

The term cherrylaurel most often encompasses three different *Prunus* species; the Carolina cherrylaurel (*P. caroliniana*), Portuguese cherrylaurel (*P. lusitanica*), and the English or common cherrylaurel (*P. laurocerasus*). Cherrylaurels are defined by their smaller but cherry-like fruit and evergreen habit. They are generally dense in form, drought tolerant, and tolerant of harsh pruning. As such, these woody shrubs
and small trees are commonly used as hedges and privacy screens in landscaping. Their 1 - 2 cm white five-petal flowers are born in upright racemes reaching from 5 - 12 cm in length. These flowers are fragrant, but not particularly pleasant in odor.

Although closely related, these three cherrylaurel species vary greatly in their native ranges. The English cherrylaurel, despite its name, is native to the black-sea region of southeastern Europe, and southwest Asia (Dirr, 2009). The more accurately name Portuguese cherrylaurel is native to the southwest coastal regions of Europe (Garcia-Verdugo et al., 2013), and the Carolina cherrylaurel native range is in the southern regions of North America (Dirr, 2009; U.S. Department of Agriculture, 2013). While all species are popular landscape plants, the English cherrylaurel is definitively the most popular.

*Prunus laurocerasus* was recorded as an introduced species to western Europe as early as 1576 (Foley and Raulston, 1994). These cherrylaurels are fast growing with large glossy leaves. This species has been cultivated for over 400 years for its ornamental qualities and ease of propagation (Dirr, 2009; Foley and Raulston, 1994). As would be expected for a plant under cultivation for so many years, there are numerous named cultivars of this species, which vary greatly in size and habit with hardiness ranging from USDA Hardiness Zone 5 – 8 (U.S. Department of Agriculture, 2016). Some of the most popular cultivars are Mount Vernon, Nana, Otto Luyken, Schipkaensis, and Zabeliana.

‘Otto Luyken’ is the most popular of the *P. laurocerasus* cultivars with a compact form and upright leaf habit. Typically, this cultivar grows between 1 – 2 m tall and 2 – 3 m wide, but taller specimens have been observed. Its dark-green leaves
generally lack the coarse serration seen in other cultivars, and stand at a distinctive upward angle. This cultivar produces abundant flowers, even in dense shade (Dirr, 2009).

‘Schipkaensis’ was discovered in a high-altitude region of Bulgaria in 1889, and in turn is one of the hardiest of cultivated *P. laurocerasus*, tolerating down to USDA Hardiness Zone 5 (Dirr, 2009). Although still considered compact, Schipkaensis is larger than Otto Luyken and has slightly larger serrate leaves. However, there is some ambiguity in this cultivar as varied forms have been observed, most notably with what is called the ‘West Coast Schipkaensis’ exhibiting an even larger, more upright form (Dirr, 2009).

Although not as popular as *P. laurocerasus*, *P. lusitanica* is also a popular landscape shrub. This species is slower growing with smaller, darker, and less glossy leaves. It is also believed to have improved drought and heat tolerance. Unlike *P. laurocerasus*, flowers form on current season’s growth rather than the previous season’s growth. As a result, *P. lusitanica* flowers in the early summer rather than early spring. Most importantly however, *P. lusitanica* is resistant to shot-hole disease, while *P. laurocerasus* is harshly, and commonly infected (Dirr, 2009).

Shot-hole disease is caused by either bacterial or fungal pathogens (e.g., *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arbicola* pv. *pruni*, *Wisonomycyes carpophilum*, *Microgloeum pruni*, and *Cercospora* spp.) (De Boer, 1980; Marchi et al., 2014; Pscheidt and Ocamb, 2014; Williams-Woodward, 1998), and presents with numerous small circular holes in the leaves of plants. These holes are caused by loss of necrotic leaf-tissue in areas killed by pathogens. This disease affects multiple
species in *Prunus*, decreasing yield of fruit crops and making ornamental species unsellable. It has been suggested that one path to shothole disease resistance in *P. laurocerasus* is through interspecific hybridization with *P. lusitanica* (Dirr, 2009).

Interspecific hybridization is a useful tool for introducing novel traits into a species of interest (Sleper and Poehlman, 2006). Within *Prunus*, interspecific hybridization is not uncommon, with one vibrant example being Pluots ® (Zaiger’s Inc., Modesto, CA), the plum-apricot hybrid. However, successful interspecific hybridization is not always possible between different species. One barrier that may stand in the way of successful hybridization, interspecific or otherwise, is a discrepancy in ploidy level.

Ploidy level, while most commonly diploid, can vary within genera (Hummer et al., 2009), and even species (Rothleutner et al., 2016) including *Prunus* (Garcia-Verdugo et al., 2013). Soltis and Soltis (1999) assert that natural polyploidization events have played a major role in the evolution of not only plants, but all eukaryotes, and that a large percentage of angiosperms have experienced a polyploidization event at some point in their evolution. More recently, Soltis et al. (2009) believe the number of angiosperms is, or nearly is 100%. Higher ploidy levels in plants are often associated gigas effects, such as shorter more compact plants with thicker, darker leaves, and larger but fewer flowers (Acquaah, 2012). Some plants have also shown increased biomass and carbon accumulation associated with an increase in ploidy (Burton and Husband, 2000; Schlaepfer et al., 2010). This could be described as an asset, as carbon fixation offsets a portion of greenhouse gas emissions during nursery production (Ingram and Fernandez, 2012).
While differences in ploidy levels can cause difficulties in breeding, they can also lead to opportunities. Artificial manipulation of ploidy level has been used to cross previously incompatible species by doubling the chromosome number of one parent to match the other (Debener et al., 2003). Furthermore, it has led to the creation of odd ploidy level plants, which are often seedless. Examples of common and economically important triploid \((2n = 3x)\) plants include seedless grapes, seedless watermelon, and bananas. In recent years, more attention has been given to the invasive potential of ornamental species (Niemiera and Von Holle, 2009), and one method to control escape from cultivation is ploidy manipulation. Olsen et al. (2006) used ploidy manipulation to create low-fertility forms of *Hypericum androsaemum* reducing their ability to escape cultivation. As such, ploidy manipulation has become a useful tool in modern plant breeding.

Using ploidy variation to one’s advantage requires the ability to measure genome size in plants of interest. Flow cytometry was developed for human cells in the mid 1960s and was first successfully applied in plants during the early 1970s to estimate nuclear DNA content (Doležel, and Bartoš, 2005). One modern form of this process extracts the nuclei of plants by finely chopping plant material in an extraction buffer to create a nuclei suspension. This suspension is stained with a DNA-specific fluorochrome which binds to the DNA in the cell. The suspension is then fed into the flow cytometer, which runs the nuclei in a single file line through a beam of UV light. The stained DNA then fluoresces, and histogram of intensity relative to genome size is produced. Genome sizes are estimated based on relative fluorescence to a known genome size, a process known as internal standardization (Doležel, 1991).
These processes are just a few of the tools available to modern plant breeders. The experiments described herein apply many of the tools and concepts mentioned above to economically important cherrylaurel species. Since *P. caroliniana*, a tetraploid (Flory, 1940), is not commonly grown in Oregon nurseries and was not a source of targeted genetic material, it was not investigated as part of these studies. The research presented herein focuses on *P. laurocerasus* and *P. lusitanica*. This work seeks to progress the knowledge surrounding these important species, and make strides to better the industry and communities that support us.
Literature Cited


Marchi, G., T. Cinelli, and G. Surico. 2014. Bacterial leaf spot caused by the quarantine pathogen Xanthomonas arboricola pv. pruni on cherry laurel in central Italy. Plant Dis. 98:1600 (abstr.).


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

Justin A. Schulze
Ryan N. Contreras
In Vivo Chromosome Doubling of *Prunus lusitanica* and Preliminary Morphological Observations

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

*Abstract.* *Prunus lusitanica* (*2n = 8x*) and *P. laurocerasus* (*2n = 22x*) 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 semi-solidified 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 (*2n = 16x*) plants, one *2n = 12x* plant, and 14 cytochimeras (*2n = 8x + 16x*) 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 (*2n = 16x*) plants displayed shorter stems, thicker leaves, and fewer but larger guard cells than the untreated controls.
Chapter Introduction

Portuguese cherrylaurel (*Prunus lusitanica*) and common cherrylaurel (*P. 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 arbicola* pv. *pruni*, *Wisonomyces carpophilum*, *Microgloeum pruni*, and *Cercospora* spp.) 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 $2n = 22x = 176$ (Meurman, 1929). While, *P. lusitanica* is an octoploid with a chromosome number of $2n = 8x = 64$ (Darlington and Wylie, 1956).

We theorize that if we can double the ploidy level of *P. lusitanica* to $2n = 16x$, 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 $2n = 19x = 152$. 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 shothole 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 $2n = 16x$ *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 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 cm × 21.6 cm × 5.2 cm flats (T.O. Plastics, Clearwater, MN) and grown in a glasshouse with day / night temperatures set to 24 °C / 19 °C. 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 µmol·m⁻²·s⁻¹ at 22 to 25 °C with a 16-h photoperiod (0600 to 0000 HR) covered with clear humidity domes (T.O. Plastics).

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 x 7 replicates x 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, approximately 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). Approximately 0.5 cm² of young leaf tissue from each sample and the internal standard was finely co-chopped 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 approximately 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 one-year post germination, 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 µm × 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 mid-vein.

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) Colour 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 Colour Chart were tested against that of the averaged L*, a*, and b* values from the leaves of the three cytotypes until ΔE* was minimized.
Data analysis. Data was analyzed using R version 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria). Survival data was non-normal with unequal variance and therefore analyzed using the non-parametric Kruskal-Wallis rank sum test (Appendix B). Treatments were then compared using least significant difference (LSD) 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 is 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. 2.1), and altered ploidy was observed in every treatment group including the controls (Table 2.1). 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 (Garcia-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 that more seedlings were required to obtain 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. 2.2). 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 \(2n = 16\times\) plants falling into the 0.2% colchicine and 125 \(\mu\text{M}\) oryzalin treatments were therefore investigated and results showed that their root ploidy levels matched that of the standard cytotype \((2n = 8\times)\), thus confirming that the alteration in ploidy for these two \(2n = 16\times\) plants was a result of our treatments.

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

We observed morphological variation among different cytotypes as the plants developed. The \(2n = 16\times\) cytotype appeared to have a more compact growth habit at one year (Fig. 2.3). When compared to the \(2n = 8\times\) cytotype, the \(2n = 16\times\) cytotype showed increased stomatal size by 43%, but decreased stomatal density by 42% (Table 2.2, Fig. 2.4). The \(2n = 12\times\) plants, although excluded from statistical analysis, fell in between the \(2n = 8\times\) and \(2n = 16\times\) 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 \(2n = 16\times\) plants displaying reduced stomatal index will hopefully exhibit the same improved drought tolerance. After 1.5 years, \(2n = 16\times\) plants exhibited shorter stems and thicker leaves than the \(2n = 8\times\) plants (Table 2.3). 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 2.4), 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 Colour 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 $\Delta E^*$ 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 cherrylarel 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 two 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*. 
Table 2.1. Sources and quantities of altered-ploidy *Prunus lusitanica* seedlings.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of treated seedlings</th>
<th>Survival rate (%)(^{z})</th>
<th>No. of Cytochimera(^{y})</th>
<th>No. of 2n = 16x</th>
<th>No. of 2n =12x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84</td>
<td>98.8 a</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>0.2% colchicine</td>
<td>84</td>
<td>98.8 a</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>0.4% colchicine</td>
<td>84</td>
<td>73.8 b</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.8% colchicine</td>
<td>84</td>
<td>60.7 b</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>125 µM oryzalin</td>
<td>84</td>
<td>16.7 c</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>420</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^{z}\)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 ≤ 0.05) differences in Kruskal-Wallis rank sum using Bonferroni-inequality adjusted least significant difference (LSD) comparisons.
Table 2.2. Stomatal density and guard cell length in three cytotypes of one-year-old *Prunus lusitanica*.

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>No. of plants</th>
<th>Guard cell length (µm)</th>
<th>Stomatal density (Stomata·mm⁻²)</th>
<th>Stomatal index²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2n = 16x</td>
<td>3</td>
<td>26.2 ± 0.61 a</td>
<td>112 ± 3.9 b</td>
<td>2938</td>
</tr>
<tr>
<td>2n = 12x</td>
<td>1</td>
<td>23.6</td>
<td>142</td>
<td>3362</td>
</tr>
<tr>
<td>2n = 8x</td>
<td>3</td>
<td>18.3 ± 0.33 b</td>
<td>194 ± 2.6 a</td>
<td>3563</td>
</tr>
</tbody>
</table>

²Stomatal index = Guard cell length • stomatal density

Values are the mean ± standard error of the mean. Letters indicate significant differences (P ≤ 0.001) using a two-sample t test. The 2n = 12x sample was excluded from statistical analysis due to lack of replication.
Table 2.3. Morphological observations of three cytotypes of 1.5-year-old *Prunus lusitanica*.

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>No. of plants</th>
<th>Plant height (cm)</th>
<th>Plant width (cm)</th>
<th>Leaf thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(n = 16x)</td>
<td>3</td>
<td>39.8 ± 1.7 b(^z)</td>
<td>40.2 ± 2.0 a</td>
<td>570 ± 4.0 a</td>
</tr>
<tr>
<td>2(n = 12x)</td>
<td>1</td>
<td>43.0</td>
<td>45.3</td>
<td>490</td>
</tr>
<tr>
<td>2(n = 8x)</td>
<td>3</td>
<td>50.3 ± 3.1 a</td>
<td>38.9 ± 3.4 a</td>
<td>470 ± 11 b</td>
</tr>
</tbody>
</table>

\(^z\)Values are the mean ± standard error of the mean. Letters indicate significant differences (\(P \leq 0.05\)) using a two-sample \(t\) test. The 2\(n = 12x\) sample was excluded from statistical analysis due to lack of replication.
Table 2.4. Leaf color values in three cytotypes of 1.5-year-old *Prunus lusitanica* collected using a colorimeter (BC-10, Konica Minolta, Ramsey, NJ)

<table>
<thead>
<tr>
<th>Ploidy</th>
<th>No. of plants</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>H°</th>
<th>C*</th>
<th>RHS Value⁷</th>
</tr>
</thead>
<tbody>
<tr>
<td>2n = 16x</td>
<td>3</td>
<td>33.1 ± 1.2 a⁵</td>
<td>-7.5 ± 0.4 a</td>
<td>13.3 ± 1.5 a</td>
<td>120.0 ± 2.0 a</td>
<td>15.3 ± 1.5 a</td>
<td>N137A</td>
</tr>
<tr>
<td>2n = 12x</td>
<td>1</td>
<td>38.0</td>
<td>-10.5</td>
<td>22.1</td>
<td>115.3</td>
<td>24.5</td>
<td>146A</td>
</tr>
<tr>
<td>2n = 8x</td>
<td>3</td>
<td>37.0 ± 2.0 a</td>
<td>-9.9 ± 0.7 b</td>
<td>20.5 ± 3.1 a</td>
<td>116.3 ± 1.6 a</td>
<td>22.8 ± 3.1 a</td>
<td>N137D</td>
</tr>
</tbody>
</table>

⁷Hue angle = H° = arctangent (b*/a*).
⁸Chroma = C* = √(a*² + b*²).
⁹Comparisons were made by minimizing ∆E* with corresponding color chart values, using the color difference equation; ∆E*₁,₂ = √((L*₂ - L*₁)² + [a*₂ - a*₁]² + [b*₂ - b*₁]²).
⁵Values are the mean ± standard error of the mean. Letters indicate significant differences (P ≤ 0.05) using a two-sample t test. The 12x sample was excluded from statistical analysis due to lack of replication.
Fig. 2.1. Flow cytometric output of *Prunus lusitanica* and induced polyploids. Over 6000 particles were run with 2\(n\) = 8\(x\), 2\(n\) = 12\(x\) and 2\(n\) = 16\(x\), plus the *Pisum sativum* ‘Ctirad’ internal standard (A). Over 3000 particles were run of one cytochimera plus the *Pisum sativum* ‘Ctirad’ internal standard (B).
Fig. 2.2. 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 $\times 200$ magnification.
Fig. 2.3. Morphological variation within and between 1.5-year-old $2n = 16x$ (A) and $2n = 8x$ (B) *Prunus lusitanica*. 
Fig. 2.4. Stomatal variation between $2n = 16x$ (A) and $2n = 8x$ (B) *Prunus lusitanica*, observed at $\times200$ magnification.
Literature Cited


Kehr, A.E. 1996. Polyploids in rhododendron breeding. J. Amer. Rhododendron Soc. 50:4


Li, W.L., G.P. Berlyn, and P.M.S. Ashton. 1996. Polyploids and their structural and


In Vitro Germination of Immature *Prunus lusitanica* Seed

Justin A. Schulze
Jason D. Lattier
Ryan N. Contreras
In Vitro Germination of Immature *Prunus lusitanica* Seed

*Additional index words.* *Prunus lusitanica*, Portugal laurel, tissue culture, embryo rescue

*Abstract.* In an attempt to hybridize *Prunus laurocerasus* and *P. lusitanica* a tissue culture protocol was developed to germinate immature *P. lusitanica* seeds in vitro. The study was conducted by first identifying the best media for germination, followed by investigating effects of seed handling. In Exp. I, seeds were collected 12 weeks after pollination (WAP) ± 1 week, and placed on media after removing the pericarp. Eight different MS media (Murashige and Skoog, 1962) were tested (M1 - M8) containing two concentrations each of BA, GA$_3$, and sucrose. The longest shoots resulted from M4 (1.45 µM GA$_3$, 6 µM BA, 30 g·L$^{-1}$ sucrose), followed by M1 (0 µM GA$_3$, 3 µM BA, 30 g·L$^{-1}$ sucrose). Radicle and shoot emergence was greater than or equal to 90% for M1 and M4. In Exp. II, M1 was used to test root and shoot emergence at 6, 9, and 12 WAP, with and without cold stratification. Little success was seen 6 and 9 WAP, with only callus development in 6 WAP, non-stratified seed. Cold stratification increased shoot emergence in the 12 WAP group from 4% to 28%, appearing to be critical for shoot emergence. If the cotyledons are retained on the seed, future efforts to expedite breeding of *P. lusitanica* using in vitro germination should not be collected prior to 12 WAP and will benefit from cold stratification before germinating on M1 or M4.

*Chemical names.* 6-benzylaminopurine (BA), gibberellic acid (GA$_3$)
Chapter Introduction

Common cherrylaurel (*Prunus laurocerasus* L.) and Portuguese cherrylaurel (*P. lusitanica* L.), collectively referred to as cherrylaurels, are highly adaptable and important ornamental species in the United States and Europe. Cherrylaurels are drought-tolerant broadleaf-evergreens commonly used in hedging. Common cherrylaurel is a mainstay in industrial and residential landscapes alike, and an important nursery crop, particularly in the Pacific northwest of the U.S. Per industry professionals however, these popular plants have one unanimous flaw, shothole disease.

Shothole disease affects multiple stone-fruit species. Symptoms present as numerous small holes in the leaves caused by fungal and/or bacterial pathogens (De Boer, 1980; Pscheidt and Ocamb, 2014; Williams-Woodward, 1998). Infection can decrease yield in fruit crops and aesthetic qualities in landscape plants. Shothole disease has plagued growers of the *P. laurocerasus* across the country, a major contributing factor being that disease severity is often intensified by overhead irrigation (Dirr, 2009; Williams-Woodward, 1998). *Prunus lusitanica*, in contrast, rarely expresses symptoms of shothole disease (Dirr, 2009).

Interspecific hybridization of these two species could provide a path to introgress shothole disease resistance from *P. lusitanica* into *P. laurocerasus* (Dirr, 2009). There have been no reports of successful hybridization of these two species. Fruit resulting from wide, interploid crosses, as in this case [*P. laurocerasus*: 2n = 22x = 176 (Meurman, 1926), *and P. lusitanica*: 2n = 8x = 64 (Darlington and Wylie,
[1956]), often abort post-fertilization (Ramsey and Schemske, 1998). One technique for recovering viable seedlings from interspecific or even intergeneric hybrids is embryo rescue.

In vitro embryo rescue has been employed in numerous genera of woody shrubs and trees including *Hibiscus* L. (Van Laere, 2007), *Prunus* L. (Kukharchyk and Kastrickaya 2006; Liu et al., 2007), *Rhododendron* L. (Eeckhaut et al., 2007), and *Rosa* L. (Gudin, 1993). Liu et al. (2007) described a technique used to rescue young embryos of peach-apricot, and peach-plum hybrids. They found the highest germination percentage occurred with the cytokinin 6-benzylaminopurine (BA), in half-strength MS basal salt media (Murashige and Skoog, 1962). Kukharchyk and Kastrickaya (2006) recovered over 500 *Prunus* hybrids in their study, and BA performed better than other cytokinins tested. Multiple sources report chilling is required in any *Prunus* species to overcome physiological seed dormancy (Moore and Janick, 1983; Kukharchyk and Kastrickaya, 2006).

*Prunus* species exhibit intermediate physiological dormancy with a chilling requirement typically 60 to 90 d, and seeds that do not receive the required dormancy period have poor, if any, germination (Hartmann et al., 2002; Moore and Janick, 1983). In the case of embryo rescue, chilling requirements will likely depend on the point in development at which abortion occurs. Seeds that have intermediate physiological dormancy accumulate abscisic acid (ABA) as they develop, which triggers the onset of dormancy (Bewley, 1997). Immature seeds low in ABA may not require cold stratification. Cold-stratification has been avoided in *Prunus* species by removal of the cotyledon and testa (Şan et al., 2014). However, it is unclear at what
point in development that these parts are distinct enough for identification and removal.

Giberellic acid (GA$_3$) is a plant hormone often associated with overcoming dormancy. This hormone was used in successful in vitro germination (Şan et al., 2014) and embryo rescue (Kukharchyk and Kastrickaya, 2006) media in Prunus, and could be an essential component to successful germination of immature seed.

Sucrose concentration is also an important media component to be considered when performing embryo rescue, as immature embryos often require lower osmotic potential than mature embryos (Trigiano and Gray, 2011).

Once in vitro germination has occurred, seedlings often require a transfer medium to continue growth (Trigiano and Gray, 2011). The stage of root and shoot development will dictate whether an in vitro seedling can be directly transplanted or first transferred to a shoot proliferation and/or rooting media. Prior research has described effective shoot proliferation and rooting media in P. laurocerasus (Kalinina and Brown, 2007; Sulusoglu and Cavusoglu, 2013). We have found no information on protocols for in vitro propagation of P. lusitanica.

During our research to improve disease resistance of P. laurocerasus by attempted hybridization with P. lusitanica minimal and brief fruit development was observed in P. lusitanica as the pollen recipient (personal observation). Therefore, we are focused our preliminary efforts to identify the optimal medium for germination of open-pollinated P. lusitanica seed. The goals of this study were to identify a tissue culture medium that would support germination of immature P.
lusitanica seed and determine the effect of collection time and cold stratification on seedling germination without removal of the cotyledon.
Materials and Methods

Media preparation. There were eight media combinations evaluated in this experiment (Table 1). The experiment was arranged in a completely randomized design with a three-way factorial treatment of means, including two levels of GA$_3$, BA, and sucrose. All media contained full-strength MS basal salts and vitamins (Murashige and Skoog, 1962). In addition, all media were amended with 100 mg·L$^{-1}$ myo-inisitol (Sigma-Aldrich, St. Louis, MO), 100 mg·L$^{-1}$ 4-morpholineethanesulfonic acid (MES) (Sigma-Aldrich), and 7 g·L$^{-1}$ agar (Sigma-Aldrich). Variable rates of GA$_3$, BA, and sucrose were added to the amended MS media (Table 1); GA$_3$ was added by filter sterilization to cooled media post autoclaving.

Prior to addition of the agar, all media was adjusted to pH 5.8 using potassium hydroxide (KOH) and hydrochloric acid (HCl). Each 150-mm culture tube was filled with 10 mL of medium and then autoclaved for 25 min at 120 °C. After the media cooled, tube caps were sealed with Parafilm M® (Bemis, Neenah, WI) until the in vitro germination procedure.

Seed collection. In Exp. I (2013), 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. Seeds were immature, and we collected just before the blush phase [approximately 12 weeks after pollination (WAP)]. In Exp. II (2015), open-pollinated seeds were collected from the same tree, but collection occurred at
approximately 6, 9, and 12 WAP. Since flowers were open-pollinated and these trees flowered for approximately two weeks, WAP was estimated ± one week.

**Seed sterilization and culture.** Fruits were collected and rinsed under running water for 10 min, moved to 70% ethanol for 30 s, and disinfected in a 20% bleach (1.65% sodium hypochlorite) (v/v), 2% Polysorbate 20 (TWEEN 20; Sigma-Aldrich) (v/v) solution for 15 min. Fruits were then triple-rinsed in nanopure, autoclaved water for 5 min per rinse. To begin in vitro germination, seeds were moved to a sterile laminar flow hood where the mesocarp (including the stony endocarp) was removed with scalpel and forceps, leaving only the immature seed. Immature seeds were cultured in the 150-mm culture-tubes, with 30 seeds per medium partially submerged. In Exp. I, after sealing tubes with Parafilm M® (Bemis), seeds were placed in a dark cooler for 10 weeks at 4 °C before being moved them to the growth area under standard growing conditions, 50 µmol·m⁻²·s⁻¹ light for a 14-hour light cycle (0600 to 2200 hr) at 25 °C. In Exp. II, all seeds were cultured in M1 with 50 seeds per collection time. Half (25) of seeds were placed for each collection time into cold stratification while the other half of seeds were placed directly in the growth area.

**Germination and morphology.** Number of seeds with emerged radicles and shoots were recorded, and shoot lengths measured 5 weeks after being placed in the growth room. Shoot length was measured to the nearest 0.5 mm, and included green stems below the cotyledon (hypocotyl), epicotyl stems below true leaves, and growth above first true leaves. Shoot length was not measured in Exp. II.
Data analysis. Data were analyzed using R-studio version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). For Exp. I, shoot length was initially analyzed including non-germinated seeds, but populations lacked normality. Thus, subsequent analysis was performed only on seedlings with emerged shoots, which normalized the population. However, this change resulted in unequal sample sizes. To account for this, least squared (LS) means were used for comparisons tests for this variable. Initially, analysis of variance (ANOVA) was performed as a three-way factorial with GA₃, BA, and sucrose as explanatory variables. However, results showed a significant three-way interaction component ($P = 0.004$), leaving interpretation difficult. Therefore, a one-way ANOVA was performed using individual media combinations (M1 – M8) as the explanatory variable, and a Tukey-Kramer adjusted least squared (LS) means comparison test was applied. Radicle and shoot emergence were analyzed using logistic regression models, with GA₃, BA, and sucrose as the independent variables (Appendix C). Model parameters for radicle and shoot emergence data analyses were tested using a likelihood ratio test. In Exp. II radicle and shoot emergence were analyzed as in Exp. I, but with WAP and cold stratification as independent variables.
Results and Discussion

Four general outcomes were seen for radicle and shoot emergence: no development, cotyledon enlargement, radicle emergence, and radicle and shoot emergence (Fig. 3.1). Radicle emergence was greater than 90% when cultured in M1 – M4 (Table 3.1). Percent shoot emergence was greater than or equal to 90% in M1, M3, and M4. The longest shoot length was observed in M4 at 42.6 mm. On average shoot length in media with 30 g·L⁻¹ sucrose was greater than with 60 g·L⁻¹ sucrose and GA had the greatest influence on shoot length at 30 g·L⁻¹ than with 60 L⁻¹ sucrose. Sucrose concentration in the media altered radicle and shoot emergence (Likelihood ratio test, P < 0.001). Radical and shoot emergence was greater on media with 30 g·L⁻¹ sucrose than with 60 g·L⁻¹ sucrose. Gibberellic Acid also significantly increased both radicle and shoot emergence (P = 0.03 and P = 0.002, respectively), but to a lesser effect.

Medium #1 was selected as the optimized medium for Exp. II. Medium #1 had an equal percentage of shoot emergence and shoot length to M4. Also, M1 did not require the additional step of filter sterilizing the GA₃, saving time, money, and contamination risk. When establishing a media for shoot proliferation in *P. laurocerasus*, previous research showed BA performing best between 1.0 – 3.0 mg/L, without using GA₃ (Sulusoglu and Cavusoglu, 2013).

In Exp. II there was 4% radicle emergence in the non-stratified group 6 WAP but no other radicle or shoot emergence among 6 or 9 WAP treatments (Table 3.2). However, the 6 WAP collection time did produce callus in two seeds in the non-
stratified group. Therefore, rescuing immature seed from as early as 6 weeks may be possible with further investigation into sub-culturing callus in this species.

Time of seed collection after pollination influenced radicle and shoot emergence (Likelihood ratio test, $P<0.001$). Cold stratification only altered shoot emergence ($P=0.02$). The 12 WAP groups were the only to produce shoots, and cold stratification increased the percentage of seeds in this group that produced shoots from 4% to 28%. It is likely that the cold stratification helped overcome germination inhibitors such as ABA, allowing for shoot emergence. Sources indicate that there are likely germination inhibitors present in the cotyledon and testa in *Prunus* (San and Yildirim, 2009). Although shoot emergence at 12 WAP with cold stratification was higher than the groups in Exp. II, it was noticeably lower than the results of Exp. I. We speculate that it was due to the presence of a testa on seed in Exp. II.

In Exp. I, seed development allowed for easy identification and removal of the testa. In Exp. II, 6 and 9 WAP seeds were too immature to identify and remove the testa without significant damage. In an effort to standardize our seed extraction procedure, the testa was not removed on the 12 WAP seed, diverging from the procedure in Exp. I. As seeds were observed in vitro, there was phenolic accumulation on the testa which was not present in seeds during Exp. I. Phenolics, combined with the possible presence of additional germination inhibitors, may be the cause of the decreased germination. Previous studies show mature *Prunus* seeds fail to germinate with intact cotyledon and testa without cold stratification; however, removal of testa from seeds showed 5% and 10% germination in apricot and peach, respectively (Şan et al., 2014). In future studies using *P. lusitanica*, perhaps
removing the cotyledons completely would allow for germination without cold-stratification or at earlier stages of development.

Introgression of shothole disease resistance from *P. lusitanica* into common cherrylaurél will likely require culturing developing, yet highly abortive, embryos in vitro to recover viable seedlings. Media developed in this study was successful in culturing immature, open-pollinated *P. lusitanica* seed at 12 WAP. The optimum germination media in this study may provide a platform for future work on embryo rescue and in vitro germination of wide cherrylaurél hybrids.
Table 3.1. Radicle emergence, shoot emergence and shoot length of immature (12 weeks after pollination) *Prunus lusitanica* seeds without testa in experiment I. Seeds underwent 10-week cold stratification and 5 weeks under standard culture conditions in eight variations of MS (Murashige and Skoog, 1962) tissue culture medium.

<table>
<thead>
<tr>
<th>Media</th>
<th>GA$_3$ (µM)</th>
<th>BAP (µM)</th>
<th>Sucrose (g·L$^{-1}$)</th>
<th>Radicle emergence (%)</th>
<th>Shoot emergence (%)</th>
<th>Seedlings (no.)</th>
<th>Shoot length (mm)$^y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0</td>
<td>3</td>
<td>30</td>
<td>100</td>
<td>90</td>
<td>27</td>
<td>$34.7 \pm 2.2$ ab$^x$</td>
</tr>
<tr>
<td>M2</td>
<td>0</td>
<td>6</td>
<td>30</td>
<td>93</td>
<td>80</td>
<td>24</td>
<td>$25.5 \pm 2.3$ bc</td>
</tr>
<tr>
<td>M3</td>
<td>1.45</td>
<td>3</td>
<td>30</td>
<td>100</td>
<td>97</td>
<td>29</td>
<td>$33.0 \pm 2.1$ bc</td>
</tr>
<tr>
<td>M4</td>
<td>1.45</td>
<td>6</td>
<td>30</td>
<td>93</td>
<td>90</td>
<td>27</td>
<td>$42.6 \pm 2.2$ a</td>
</tr>
<tr>
<td>M5</td>
<td>0</td>
<td>3</td>
<td>60</td>
<td>63</td>
<td>47</td>
<td>14</td>
<td>$25.5 \pm 3.0$ bc</td>
</tr>
<tr>
<td>M6</td>
<td>0</td>
<td>6</td>
<td>60</td>
<td>47</td>
<td>33</td>
<td>10</td>
<td>$26.3 \pm 3.6$ bc</td>
</tr>
<tr>
<td>M7</td>
<td>1.45</td>
<td>3</td>
<td>60</td>
<td>70</td>
<td>60</td>
<td>18</td>
<td>$27.6 \pm 2.7$ bc</td>
</tr>
<tr>
<td>M8</td>
<td>1.45</td>
<td>6</td>
<td>60</td>
<td>80</td>
<td>70</td>
<td>21</td>
<td>$22.2 \pm 2.4$ c</td>
</tr>
</tbody>
</table>

$^x$Number represent seeds with emerged roots and shoot, and serves as sample sizes (n) used for performing the data analysis on shoot length.

$^y$Least squared mean ± LS standard error of the mean.

$^x$Mean separation based on Tukey-Kramer adjusted LS means comparison test with $P \leq 0.05$. 
Table 3.2. Radicle emergence and shoots emergence of immature *Prunus lusitanica* seeds with testa cultured at three different collection times in experiment II. Seeds were evaluated with and without 10-week cold stratification and 5 weeks under standard culture conditions.

<table>
<thead>
<tr>
<th>WAP(^z)</th>
<th>Culture method</th>
<th>Radicle emergence (%)</th>
<th>Shoot emergence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Non-stratified(^y)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Cold stratified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Non-stratified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Cold stratified</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>Non-stratified</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>Cold stratified</td>
<td>28</td>
<td>28</td>
</tr>
</tbody>
</table>

\(^z\)Weeks after pollination (WAP)

\(^y\)Two seeds in this group produced callus.
Fig. 3.1. Different outcomes of in vitro germination of cold-stratified and direct sown *Prunus lusitanica* collected 6, 9, and 12 weeks after pollination: (A) No development, seen in most of the 6 and 9 weeks after pollination (WAP) groups, (B) cotyledon enlargement but no radicle or shoot emergence, seen in both 12 WAP groups, (C) radicle emergence with no shoot emergence, common in the 12 WAP direct culture group, (D) radicle and shoot emergence as seen in 28% of 12 WAP cold-stratified group.
Literature Cited


Comparing Vegetative Propagation of Two ‘Schipkaensis’ Common Cherry Laurel Ploidy Levels

Justin A. Schulze
Ryan N. Contreras
Carolyn F. Scagel

HortTechnology
ASHS 1018 Duke St. Alexandria, VA 22314
In press
Comparing Vegetative Propagation of Two ‘Schipkaensis’ Common Cherrylaurel Ploidy Levels

*Additional index words.* polyploidy, *Prunus laurocerasus*, semi-hardwood cuttings, IBA, NAA

*Abstract.* Common cherrylaurel (*Prunus laurocerasus*) ‘Schipkaensis’ is an important nursery crop across the United States. In our breeding efforts to reduce shothole symptoms and weediness, we have created chromosome doubled forms of this cultivar. Vegetative propagation is an important factor in nursery production, and we have found no studies that have looked at comparative adventitious rooting of stem cuttings using induced polyploids. The objective of this research was to determine if rooting ability varied between these two ploidy levels. Semi-hardwood stem cuttings from wild-type (22x) and polyploid (44x) ploidy levels were taken at the end of July 2015 and the beginning of July 2016. Cuttings were dipped in 1030 ppm (0.10%) Indole-3-butyric acid (IBA) and 660 ppm (0.066%) 1- Naphthaleneacetic acid (NAA) before being set in rooting substrate. After one month, cuttings were removed from substrate and data collected. Data included; rooting percentage, root number per rooted cutting, average root length, and total root length. In 2015, 88% of the cuttings from the 44x plants and 63% of the cuttings from the 22x plants rooted. In 2016, 100% of cuttings from both ploidy levels rooted. In both years, average root length and total root length were similar between ploidy levels; however, cuttings from 22x plants generally had more roots than those from 44x. Chromosome doubled
‘Schipkaensis’ common cherrylaurel rooted effectively, and produce transplantable cuttings similar to the standard ploidy.
Chapter Introduction

Common cherrylaurel is a species of evergreen stone fruit native from Eastern Europe to Southwest Asia. In some parts of the Mediterranean, this species is grown for food and medicinal purposes (Kolayli et al., 2003). In the United States and Europe, cultivars of this species are common ornamental landscape plants. Popular compact cultivars include; Mount Vernon, Otto Luyken, Schipkaensis, and Zabeliana. Many cultivars are susceptible to bacterial and fungal pathogens (e.g., *Pseudomonas syringae* pv. *syringae*, *Xanthomonas arbicola* pv. *pruni*, *Wisonomyces carpophilum*, *Microgloeum pruni*, and *Cercospora* spp.) that cause shothole disease (De Boer, 1980; Marchi et al., 2014; Pscheidt and Ocamb, 2014; Williams-Woodward, 1998). Shothole disease presents with numerous small circular holes in the leaves of plants, these holes are caused by loss of necrotic leaf-tissue in areas killed by pathogens. This can greatly reduce ornamental appeal, and in severe cases, cankers that girdle stems may kill infected plants. Due to abundant fruit production, this species has become naturalized across areas of the North American west coast (U.S. Department of Agriculture, 2006), and in other regions is considered invasive (Hättenschwiler and Körner, 2003).

In attempts to address shothole symptoms and the weedy tendencies in this species, we created chromosome-doubled forms of ‘Schipkaensis’. While many studies have compared morphological variability in ploidy series (Huang et al., 2015; Kermani et al., 2003; Li et al., 1996; Ulrich and Ewald, 2014), we have found none that addressed adventitious rooting of stem cuttings. Successful vegetative
propagation is an important consideration in determining plant potential for large scale production (Hartmann et al., 2011). We wanted to assess if alteration in ploidy level had an effect on vegetative propagation of this plant.

Previous research seeking to optimize vegetative propagation of common cherrylaurel successfully rooted hardwood cuttings at high percentages (Ribeiro, 2010; Sülüşoğlu and Çavuşoğlu, 2009; Yazıcı, 2009). Ribeiro (2010) reported exogenous application of the auxin Indole-3-butyric acid (IBA) improved rooting percentage compared to a control without IBA, but IBA ranging from 1000 ppm to 7500 ppm (0.1% to 0.75%) produced no statistically significant difference among treatments. Other sources report taking semi-hardwood cuttings in summer is also effective (Adams, 1983; Dirr, 2009; Sülüşoğlu and Çavuşoğlu, 2010). The cultivar Schipkaensis, in particular, has been reported to root as quickly as 3 weeks (Adams, 1983). Our objective was to determine if rooting percentage and other root traits vary between natural \([2n = 22x =176 \text{ (Meurman, 1929)}]\) and chromosome doubled \((2n = 44x = 352)\) ploidy levels of ‘Schipkaensis’ common cherrylaurel.
Materials and Methods

*Plant material.* In 2015, standard ploidy level (22x) ‘Schipkaensis’ common cherry laurel material was collected from 3-year-old commercially grown plants (Blue Heron Farms, Corvallis, OR) growing in 3-gallon containers. Chromosome doubled (44x) plant material was confirmed via flow cytometry (Contreras and Meneghelli, 2016), and collected from three 3-year-old plants growing in 7-gallon containers containing a soilless fir-bark substrate. The 44x plants received 20N-4.4P-8.3K controlled release fertilizer (Apex ® Evergreen, J.R. Simplot, Boise, ID) at a rate of 32 g per container annually in April. All 44x plants were under overhead irrigation twice per day for 40 min when cuttings were collected. In 2016, cuttings were collected from the previous year’s rooted cuttings (1-year-old) growing in 3-gallon containers, under the irrigation and fertility conditions previously stated. All plants in both years were grown on outdoor container pads in full sun.

*Cutting treatments.* On 29 July 2015 and 5 July 2016, 24 cuttings from each ploidy level were taken. Cuttings were 7 to 10 cm long with three to five nodes. The three youngest leaves were retained on cuttings and any remaining leaves were removed. Retained leaves were bisected to reduce water loss. The basal 5 cm of cuttings were dipped for 10 s in 1030 ppm IBA (0.1%) and 660 ppm (0.066%) 1-Naphthaleneacetic acid (NAA) dissolved in 9.8% isopropyl alcohol (Woods Rooting Compound, Earth Science Products, Wilsonville, OR) and the lower 3 to 5 cm of each cutting was inserted into 4-inch square containers (Gage 400s, Merril’s Packaging, Burlingame, CA) filled with 2 perlite (Supreme Perlite, Portland, OR): 1 soilless
substrate (Metro-Mix 840PC; SunGro Horticulture, Agawam, MA) (by volume). Each year, the 48 cuttings were randomly arranged in three 17-inch square flats, with eight replicates from each ploidy level in a flat. Flats were placed in a clear 6 mils polyurethane mist-tent with bottom heat (78 °F) under intermittent mist (30 s every 30 min from 0600 HR to 2000 HR) for 1 month. The mist-tent was located in a glass-house covered with a 50% shade cloth and temperatures set to 75 °F day/65 °F night. The glass-house was located on the campus of Oregon State University in Corvallis, OR.

*Rooting evaluation.* Cuttings were removed from the substrate, and rinsed gently with water to remove remaining substrate. Cuttings were placed on a flat surface and the roots were manually spread apart. Roots on each cutting were measured using a standard metric ruler to the nearest millimeter. Number of roots and length of each root on all rooted cuttings were measured if the root was over 2.0 mm long. The total root length and average root length was calculated per cutting.

*Data analysis.* Data was analyzed using R-studio version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). The experimental design was a randomized complete block design with replication. Due to lack of normality and unequal variance, rooting percentages were compared using the non-parametric Kruskal-Wallis rank sum test (Appendix D). Year and ploidy were evaluated separately, with flats as experimental units. Furthermore, the dependent variables; root number and total root length, failed a Levene’s test for homogeneity of variance, and were subsequently transformed using a square root transformation. After transformation, variances were stabilized, and analysis was performed for root length,
root number, and total root length using a two-way analysis of variance (ANOVA).
This portion of the analysis considered ploidy and year explanatory variables, and experimental units were individual cuttings. $P$ values were determined using type III marginal sum of squares to account for unequal sample size resulting from several unrooted 2015 cuttings.
Results and Discussion

Ploidy had no influence on the number of cuttings that rooted in either year (Fig. 4.1A). In 2015, 63% of the 22x cuttings rooted and 88% of the 44x rooted, but the variability among replicates was high resulting in similar rooting between 44x and 22x plants. All cuttings rooted in 2016. Previous research of wild-type common cherrylauré cuttings resulted in rooting between 60% and 88%, when IBA was used (Ribeiro, 2010; Sülüşoğlu and Çavuşoğlu, 2009, 2010). Our results are consistent with Dirr (2009), who reports up to 100% rooting in semi-hardwood cuttings taken in summer.

Increased ploidy decreased the number of roots produced by cuttings (Fig. 4.1B). On average, cuttings from 22x plants produced 21 roots and 44x plants produced 13 roots, representing a 38% decrease in root number. Having fewer roots could have a detrimental effect on water and nutrient absorption. However, in our study we applied only one concentration of IBA and NAA. Sülüşoğlu and Çavuşoğlu (2010) show an increase in number of roots from 1000 to 2000 ppm (0.2%) IBA, and then subsequent decrease in root number from 2000 to 8000 ppm (0.2% to 0.8%) IBA. The 44x plants may have different requirements for optimal rooting compared to 22x, and perhaps altering auxin concentrations could improve the root number on 44x cuttings. Root number also varied by year, suggesting sensitivity to factors other than auxin-concentration and ploidy, such as collection time. In previous studies, collection time appears to affect root number per cutting in this species. Hardwood cuttings taken in March (Sülüşoğlu and Çavuşoğlu, 2009), and semi-hardwood
cuttings taken in July (Sülüşoğlu and Çavuşoğlu, 2010), both treated with 2000 (0.2%) ppm IBA, produced an average of 55.0, and 22.2 roots per rooted cutting, respectively.

Ploidy did not influence average root length (Fig. 4.1C). Average root length of cuttings from 22x plants (20.2 mm) were similar to that of 44x plants (18.5 mm) for both years. However, average root length in 2016 was 25% greater than in 2015. This suggests that collection timing may affect average root length as well as number of roots per cutting. Compared to our results, Sülüşoğlu and Çavuşoğlu (2010) reported longer average root length (42.2 to 62.6 mm) on cuttings of wild-type common cherrylauré evaluated after 90 d. It is likely that increasing time to evaluation would also increase our average root length.

Total root length per cutting did not differ between years (Fig. 4.1D). On average, total root length on cuttings from 22x plants (37.8 cm) was greater than total root length on cuttings from 44x plants (22.6 cm), but this difference was not statistically significant.

Our study confirmed the chromosome doubled form of ‘Schipkaensis’ common cherrylauré roots at a sufficiently high percentage and produces transplantable cuttings (Fig. 4.2). Compared to the 22x ploidy level, the 44x produced fewer roots, but this difference is not likely commercially important. Visually, the root systems on cuttings of both ploidy levels were coarse with no branching or fine root development. Roots of 44x cuttings did appear slightly thicker, but we did not measure root diameter or determine root dry weight. Future studies should include these factors when assessing differences in rooting among treatments. All rooted
cuttings have been successfully transplanted into larger containers, and we have seen no negative effects of reduced root number, even though the 44x cuttings produced nearly 10 and 5 fewer roots per cutting in 2015 and 2016, respectively. As propagation can be a bottleneck for production, it is important to ensure that new cultivars are able to be propagated efficiently. In our study, there were no apparent detrimental effects to rooting cuttings of ‘Schipkaensis’ common cherrylaural following induction of higher level polyploids.
Fig. 4.1. Adventitious rooting (%), average root length, root number, and total root length on stem cuttings from 22x and 44x ‘Schipkaensis’ common cherrylaurel. Cuttings were taken in late July 2015 and in early July 2016. (A) Column means and standard error bars based on Kruskal-Wallis rank sum test (n=6). (B, C, D) Unrooted samples in 2015 resulting in variable sample size (2015 22x: n=15, 2015 44x: n=21, 2016 22x: n=24, 2016 44x: n=24). (B, D) P values for ploidy and year are for main effects from ANOVA, using transformed variables. Columns are back-transformed means and error bars are one standard error of the mean. (C) P values for ploidy and year are for main effects from ANOVA. Columns are least squares (LS) means and error bars are one standard error of LS means; 1 mm = 0.0394 inch, 1 cm = 0.3937 inch.
Fig. 4.2. Rooted stem cuttings from 22x and 44x ploidy levels of ‘Schipkaensis’ common cherrylaurer. This photo was taken of 2016 cuttings that measured the highest in the category “total root length” from each block; 1 cm = 0.3937 inch.


Dirr, M.A. 2009. Manual of woody landscape plants: Their identification, ornamental characteristics, culture, propagation and uses. 6\textsuperscript{th} ed. Stipes Publishing, Champaign, IL.


Chromosome Doubling of *Prunus laurocerasus* ‘Otto Luyken’ Altered Morphophysiology and Reduced Carbon Fixation in Container Production

Justin A. Schulze
Ryan N. Contreras
Carolyn F. Scagel
John G. Lambrinos

Journal of American Society for Horticultural Science
ASHS 1018 Duke St. Alexandria, VA 22314
To be submitted
Chromosome Doubling of *Prunus laurocerasus* ‘Otto Luyken’ Altered Morphophysiology and Reduced Carbon Fixation in Container Production

*Additional index words.* Common cherrylaurel, flow cytometry, polyploid, photosynthesis, water-use efficiency, stomatal conductance.

*Abstract.* *Prunus laurocerasus* ‘Otto Luyken’ is a popular, and economically important landscaping shrub across the United States. Chromosome-doubled cytotypes have been created in efforts to produce sterile forms. This study compared physiological and morphological characteristics of tissue culture sourced chromosome doubles cytotype (TC-44x; 2*n* = 44x), tissue culture sourced standard cytotype (TC-22x; 2*n* = 22x) and a nursery sourced standard cytotype (N1-22x; 2*n* =22x). All plants were planted into 26.5-L containers and provided the same irrigation and fertility during the study. Plant height and width was measured before the initial growing season (T0), after the first growing season (T1) and after the second growing season (T2). At T1 and T2 plant biomass and tissue nutrient composition were also determined. In almost all cases, the TC-44x plants were smaller in height and width than both TC-22x and N1-22x. Few differences were seen in the nutrient, water-content and carbon-content of harvested plants. We estimated that a single TC-44x plant fixed significantly less carbon than TC-22x in both years. In attempts to ascertain the differences in ability to fix carbon during the second growing season, we measured leaf photoassimilation on several dates. Leaves of the TC-44x plants were performing photoassimilation at the same rate as TC-22x,
but had increased stomatal conductance and transpiration. Water-use efficiency was significantly lower in TC-44x than all other plant categories. We believe decreased water-use efficiency may have contributed to the discrepancy in carbon fixation.
Prunus laurocerasus, known as common cherrylaurel or English laurel, is a densely growing evergreen tree or shrub with many cultivars of varying growth habits. These cultivars can range from 1 to 8 m tall (Dirr, 2009). The most popular cultivar is ‘Otto Luyken’, which is a low growing compact cultivar that is widely used in landscapes. This cultivar grows to 1 to 2 m high and spreads 2 to 3 m. Its leaves are smaller and finer textured than the species (2.5 cm x 10 cm) and generally lack the coarse serration. Additionally, ‘Otto Luyken’ flowers prolifically even in dense shade but tends to produce fewer fruit than wild-type (Contreras, personal observation).

Prunus laurocerasus has a high chromosome number (2n = 22x = 176) that may lead to aneuploidy among individuals (Meurman, 1929). Aneuploidy has not been confirmed but it does not appear to influence fertility if it is present. Due to the heavy fruit production of this species which are spread by birds, it is often considered weedy, and has become naturalized across much of the western United States (U.S. Department of Agriculture, 2006). In some regions, P. laurocerasus is even considered invasive (Hättenschwiler and Körner, 2003). In attempt to develop odd-ploidy cultivars (e.g. 33x) with reduced fertility, Contreras and Menghenelli (2016) used in vitro polyploidization to develop 2n = 44x = 352 P. laurocerasus ‘Otto Luyken’ plants. Currently, we hope to investigate the 44x cytotype to better understand its morphophysiological relationship to the standard cytotype.
For induced polyploids to be useful in the nursery industry they must first be easy to propagate. Chromosome doubled cytotypes of *P. laurocerasus* ‘Schipkaensis’ have been reported to root with similar efficiency to the standard cytotype (Schulze et al., In press.). Another obvious stipulation of nursery production, is that polyploids must have increased or at least equivalent aesthetic value as the standard cytotype. Increased ploidy plants tend to have broader and thicker leaves, darker colored foliage, increased stomatal size, and decreased stomatal density compared to standard cytotype plants (Kermani et al., 2003; Gu et al., 2005; Liu et al., 2007; Schulze and Contreras, In press.; Ye et al., 2010). Ye et al. (2010) and Gu et al. (2005) also observed increased inflorescence size. Kermani et al. (2003) observed double the number of petals per flower of tetraploids compared to diploids in work with *Rosa*. These observations do not rule out these plants for nursery production, and in some cases, show potential for improved ornamental value.

Li et al. (1996) studied diploids and natural polyploids of *Betula papyrifera*. They found that decreased stomatal density along with increased epidermal thickness of polyploids contributed to increased drought tolerance and water conservation. Another study reported an increased ploidy level increased photosynthetic efficiency as it relates to water stress in *Lonicera japonica* (Li et al., 2009). Some naturally occurring polyploids of herbaceous perennials have shown increased biomass accumulation compared to diploid forms (Burton and Husband, 2000; Schlaepfer et al., 2010). However, few studies to date have looked at biomass accumulation of woody species as related to ploidy level. We plan to look at biomass accumulation in
terms of carbon fixation and its potential to offset a portion of emissions in nursery production.

Carbon fixation and long-term sequestration has the potential to offset a portion of greenhouse gas (GHG) emissions related to nursery production. To exemplify this potential, in 2001 it was estimated that urban trees in the USA stored 700 billion kilograms of carbon, and have an annual carbon sequestration rate of 22.8 billion kilograms per year (Nowak and Crane, 2001). With this in mind, Life Cycle Assessments (LCA) of nursery production are an important tool that can quantify environmental impact of producing plants, in which carbon fixed by plants offset a portion the GHG cost (Ingram and Fernandez, 2012). Such an LCA was performed on the broadleaf evergreen *Ilex crenata* (Ingram et al., 2016), displaying this potential.

Accounting for carbon budget could establish plant-breeding practices with the objective of addressing climate change. We suspect plants that fix more carbon would have improved marketability by responding to consumer demand for sustainably produced products, in addition to the benefits of reducing GHG emissions across the nursery industry. Biomass accumulation has been studied between species (Prior et al., 2011), but there is little information about biomass accumulation in woody species with respect to ploidy level. However, variation in traits such as drought tolerance and photosynthetic efficiency have been observed in woody species based on ploidy level (Zwack et al., 1998; Li et al. 1996; Li et al., 2009). Perhaps these types of variations could alter biomass accumulation, and in turn carbon
fixation. Although the plants in this study were developed for a different reason, we hope to explore all effects of chromosome doubling to better understand these plants.

General allometric equations are often used to estimate the standing biomass of woody plants (Chojnacky et al. 2013). However, the currently available equations are not well suited to estimating biomass across the diverse range of woody nursery crops. Attempts have been made to modify standard practice to accommodate other plant forms. For example, Navar et al. (2004) considered each stem of a multi-stemmed shrub its own plant, and calculated stem width and height for each. Uso et al. (1997) estimated the aboveground biomass of Mediterranean shrubs by estimating a whole plant cylindrical volume, requiring only height and width measurements. A portion of the plant that is not addressed by these allometric equations is root biomass. Using container-grown plants to determine allometric equations in a production setting would lend the ability to account for carbon fixed in the root systems. Carbon that is stored as root biomass can be a significant portion of a plant's total sequestered carbon. Roots provide long term storage of carbon in soil, even after the plant is removed from the environment. Larger, deeper root systems could positively impact carbon sequestration to soil while improving water uptake and drought tolerance (Jansson et al., 2014).

The growing interest in environmental friendliness, and demand for products that embrace these ideas, have led to new directions in horticultural research. Comparing plant biomass accumulation between chromosome-doubled plants and the standard cytotype may prove valuable. If *P. laurocerasus* ‘Otto Luyken’ with doubled chromosomes could effectively fix more carbon, then it would display a
reduced carbon footprint during production and beyond. These combined effects could improve marketability while simultaneously reducing environmental impact.

The goals of this study are to (1) quantify differences in carbon fixation between two *P. laurocerasus* ‘Otto Luyken’ cytotypes, (2) to use morphological and physiological measurements to explain why differences in carbon may occur between cytotypes, (3) and evaluate the ornamental qualities of the two cytotypes.
Materials and Methods

Plant material. There were four categories of plants in this experiment, the first two are the chromosome doubled (TC-44x), and standard-cytotype (TC-22x) *P. laurocerasus* ‘Otto Luyken’. These were the result of an in vitro chromosome doubling experiment (Contreras and Menghelli, 2016), where the TC-22x served as the control plants. These plants have been grown under overhead irrigation in full sun with winter protection since their propagation in 2013. The other two plants types are 3.8-liter nursery-sourced (Blue Heron Farm; Corvallis, OR) standard-cytotype (N1-22x), and 11.4-liter nursery-sourced (Blue Heron Farm) standard-cytotype (N3-22x) *P. laurocerasus* ‘Otto Luyken’. Both types were donated by the nursery where they were being grown if full sun with overhead irrigation. The N1-22x and N3-22x were used as a reference for size similarity (N1-22x) and age similarity (N3-22x) to the TC-22x and TC-44x plants.

At the start of the experiment there were 23 TC-44x, 11 TC-22x, 30 N1-22x, and five N3-22x. The TC-44x and TC-22x plants had been grown in the same conditions prior to the onset of the study. All the TC-44x plants, and subsets of the standard-cytotype categories were confirmed via flow cytometry as per Contreras and Meneghelli (2016) at the beginning of each growing season. All plants were repotted into 26.5-liter containers with soilless media (Patio Potting Soil™; Rexius, Eugene, OR) containing 76% fir bark (< 1.9 cm), 9% pumice, 7.5% garden compost, 4.5% manure (horse, chicken and steer), 2.5% peat, and 0.5% perlite. All plants and supplied with 20N-4.4P-8.3K controlled release fertilizer (Apex® Evergreen, J.R.
Simplot, Boise, ID) at a rate of 32 g per container, annually in April. All plants were arranged in a completely randomized design on a nursery pad in full sun with spray stake irrigation daily at 0800 HR and 1600 HR, and maintained at an approximate 20% leaching fraction. Plants remaining after destructive harvest were moved to an unheated polyhouse from late-December to mid-April.

*Morphology measurements.* Plant height, and width were measured using a standard ruler to the nearest 0.5 cm. For these measurements, if flower height exceeded the height of the plant canopy or furthest width of the canopy it was not included in measurements. For plant width, the plant’s widest spread was measured, followed by the spread perpendicular to the widest spread, and then these two measurements were averaged. Plant volume was estimated based on the volume of a cone, with average plant width serving as conical diameter, and plant height serving as conical height. Morphology measurements were taken in early May 2015 before the first growing season of this study (T0), in late December 2015 after the first growing season (T1), and in late November 2016 after the second growing season (T2). The TC-22x, TC-44x and N3-22x were approximately 3-years-old at the start of the study, while N1-22x was approximately 1-year-old.

*Destructive harvests.* Plants were destructively harvested at T0, T1 and T2 after morphological measurements were taken. Three plants from every category were harvested at T0. The N3-22x plants were only harvested at T0. For the remaining harvests, three plants from every category were harvested, except for T2 in which only one TC-44x was harvested. At each harvest, plants were cut at soil level, then shoots were rinsed with running water. Roots were washed separately to remove
all media from the root system. Plants were then separated into the following parts; roots, stems > 5 mm (large stems), stems < 5 mm (small stems), green stems, current season’s leaves (new leaves), past seasons’ leaves (old leaves), and fruits + flowers. After plant parts were separated, they were placed into paper bags, fresh weight was recorded to the nearest 1 g, then were dried in an oven set to 60 °C. After all water was removed, dry weight was recorded for all parts to the nearest 1 g. Water content (%) was calculated for all parts except roots by subtracting dry weight from fresh weight, and then dividing by fresh weight. Root to shoot ratios were recorded for every harvested plant by dividing dry weight of the root systems by the dry weight of aboveground biomass (excluding flower and fruit).

*Tissue analysis.* All dried plant materials were ground to pass through a 1 mm screen. Percentages of carbon and nitrogen per sample were determined using a combustion analyzer (TruSpec CN; Leco Corp., St. Joseph, MI). Other nutrients were analyzed using an Inductively Coupled Plasma-Optical Emission Spectroscopy (Optima 3000DV; Perkin Elmer, Wellesley, MA) following microwave digestion in 70% (v/v) nitric acid (Gavlak et al., 2003). Total plant content of each nutrient was calculated as the sum of the nutrient content from each plant organ. Calculations were performed on all harvested plants but reported only for the T1 and T2 harvests, as plants at T1 were exposed to identical fertility treatments and growing environment for at least one full growing season.

*Carbon fixation.* Multiple linear regression was used to test the hypothesis that plant volume and category could predict whole-plant biomass. An allometric equation was established using the best fit line of this regression from measurements...
of all the harvested plants, except for the three N3-22x which were only to serve as a reference and only harvested at T0. Using this equation, volumes of all plants were converted to predicted-biomass. Then, weighted-carbon (%) was multiplied by the predicted-biomass to estimate carbon content. The amount of annual carbon fixed was estimated as the biomass increment between T1 and T2 by subtracting estimated carbon at T0 from T1 and from T1 to T2.

Leaf gas exchange. In summer between T1 and T2, one young fully expanded young leaf of all plants were tested on seven separate days using a portable photosynthesis system (LI-6400XT; LI-COR Inc., Lincoln, NE). Measurements were taken on clear days between 1100 and 1300 HR using the clear leaf chamber with the flow meter set to 400 µmol·s⁻¹, the CO₂ mixer set to 400 ppm, and the temperature and relative humidity set to ambient conditions. Temperatures at time of collection ranged from 22 to 32 °C. Leaf photoassimilation rate (µmol·m⁻²·s⁻¹), stomatal conductance (mol·m⁻²·s⁻¹), transpiration (mmol·m⁻²·s⁻¹), and water-use efficiency (photoassimilation rate/ transpiration) (mmol·mol⁻¹) were calculate from gas exchange measurements. Measurements were grouped into three temperature categories, 23 – 27 °C, 27 – 30 °C, and 30 – 32 °C, with at least two subsamples per range.

Stomatal measurements. Shortly before T2, length of stomatal guard cells was measured using a compound light microscope (Zeiss Imager A1, Zeiss Microscopy, Oberkochen, Germany) for all TC-44x and subsets of the remaining standard cytotype plants. Three fully expanded leaves were randomly selected and only the abaxial leaf surfaces were used for microscopic observation, as no stomata were observed on the
adaxial surface of the leaf. 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. By using an image analysis software (AxioVision; Zeiss Microscopy), 150 stomata per plant were measured at \( \times 100 \) magnification. The same imaging software with the same magnification setting was then used to count stomata per frame of view (673.1 \( \mu \text{m} \times 897.4 \mu \text{m} \)), and determine stomatal density (stomata·\( \text{mm}^{-2} \)). Fifteen images per plant were analyzed to determine stomatal density.

\textit{Data analysis.} Data was analyzed using SAS statistical software 9.4 (SAS Institute, Cary, NC). Sample sizes were unequal for many of the analyses. Therefore, in such cases least squared (LS) means were used for reporting means, and for mean comparison tests. PROC MIXED with the “repeated” statement was used for analysis of variance (ANOVA) in measurements that used the same plant multiple times (Appendix E). Repeated measures were taken in gas exchange measurements and for carbon fixation measurements. Plant-volume data were log transformed and carbon-fixation data were square-root transformed for analysis meet ANOVA assumptions. These data were reported after being back-transformed. Tukey-Kramer honestly significant difference (HSD) test was used when comparing size data of plant categories. Multiple linear regression was used to compare biomass by volume and category to create a biomass prediction equation.
Results and Discussion

The experiment began with 24 confirmed TC-44x, 11 TC-22x, 25 N1-22x, and 5 N3-22x. The four types of plants varied significantly in height width and volume (Fig. 5.1A-B). The N3-22x plants were included in the study as a size reference because they were approximately the same age as the tissue culture plants (TC-22x and TC-44x). It is likely, being sourced from tissue culture and differences in growing methods contributed to the difference in size. The N1-22x plants were selected based on approximate similarities in size, and were used as a reference to nursery grown P. laurocerasus ‘Otto Luyken’ throughout the remainder of the study. At the start of the experiment, the TC-44x plants had the smallest in width and volume of all plant categories.

During the study, we observed at high percentage of ploidy reversion in the TC-44x plants. Between our initial and subsequent screening of genome sizes, we observed a reversion of 81% to the 2n = 22x state in TC-44x. Reversion of induced polyploids to initial ploidy level is often observed (Hamill et al., 1992; Lattier et al., 2013; Lavania, 2005). Reversion occurred from 2n = 44x to 2n = 22x + 44x, 2n = ~33x, and 2n = 22x forms. We speculate reversion of higher level polyploids occurred because of diplontic selection (Klekowski, 2003), where even a single cell, when able to divide more quickly, can out compete neighboring meristematic cells, and eventually convert whole shoots to the diploid state. In the case of this study, we speculate the natural ploidy (2n = 22x) cells were present in undetectable amounts during the initial screening process, and as plants grew, these cells were able outgrow
the larger, more slowly dividing chromosome-doubled \((2n = 44x)\) cells. Furthermore, we speculate that this may have occurred at such a high rate because of the already redundant wild-type genome of this species, and other research suggests a relationship between higher ploidy levels and higher rates of reversion (Lavania, 2005). It appears these plants may be difficult to maintain in the chromosome-doubled state, which would hinder production. Reverted plants were excluded from all calculations of the study.

Throughout the experiment, the TC-44x plants maintained a smaller form compared to the standard cytotype plants. At T1, TC-44x plants had the smallest width, and volume (Fig. 5.1A-B; Fig. 5.2). At T2, width and volume in TC-44x plants were smaller than TC-22x plants, but similar to N1-22x plants (Fig. 5.1A-B). Throughout the study, TC-44x plants were shorter than other plant types. Compact form is common in chromosome-doubled cytotypes when compared to wild-type (Huang et al., 2015; Liu et al., 2007; Schulze and Contreras, In press.). This could be a benefit in commercial production as it is likely that these shorter plants would require less pruning.

Categories had similar dry-weight proportions of plant parts, with the exception weight of fruit and flowers (Fig. 5.3). The TC-44x plants had not yet flowered and as such no biomass of fruit and flowers was produced. In contrast, all the TC-22x and N1-22x had flowered by T1 or earlier. For the remainder of the study, fruit and flower weights have been excluded from calculations since such weight would not contribute to long-term carbon fixation within these plants. Water-content, root to shoot-ratio, carbon, phosphorus, and potassium were not different
between plant categories (Table 5.1). Nitrogen was significantly higher in the TC-44x than N1-22x but not higher than TC-22x. Perhaps an increase in nitrogen could be related to the presence of twice the amount of DNA in the genome of the chromosome-doubled plants, but also appears to have been altered by lingering cultural effects as well.

Category was not a significant factor when modelling regression of biomass in terms of plant volume. Therefore, the best-fit linear trend-line \( y = 0.0132x + 22.58 \), with the x-variable representing conical volume, served as the allometric equation for estimating whole-plant biomass of all plants (Fig. 5.4).

We estimated that a single TC-22x plant fixed significantly more carbon than TC-44x during both growing seasons (Fig. 5.5). We believe ploidy level was the most important factor contributing to the decrease in carbon fixation of the \( 2n = 44x \) cytotype. Median carbon fixation was also significantly lower in the first growing season (T0 - T1), than the second (T1 - T2). We believe a combination of increased plant size and continued adaptation to a new growing environment played a role in the large difference seen in the two years.

Stomatal density was not significantly different between TC-22x and N1-22x, but both were significantly higher than TC-44x (Table 5.2, Fig. 5.6). Also, TC-44x guard-cell length was significantly larger than both TC-22x and N1-22x. Interestingly, although the difference is much less, guard-cell length was significantly smaller in N1-22x than TC-22x. We believe that this variation may be due to the differences in growing environment prior to the experiment. Differences in guard-cell length and stomatal density represent a 35% increase, and a 50% decrease,
respectively, resulting from chromosome doubling in this cultivar (TC-22x to TC-44x). These trends agree with stomatal measurements previously observed in chromosome-doubled *P. lusitanica* (Schulze and Contreras, In press.), and chromosome doubled forms of other species such as *Lobularia maritima* (Huang et al., 2015) and *Lagerstroemia indica* (Ye et al., 2010).

Even with significantly reduced stomatal density, average stomatal conductance for the TC-44x leaves (0.22 ± 0.012 mol·m⁻²·s⁻¹) was significantly higher than both TC-22x (0.19 ± 0.009 mol·m⁻²·s⁻¹) and N1-22x (0.16 ± 0.005 mol·m⁻²·s⁻¹) (Table 5.2). We also observed leaves of the TC-44x plants had increased transpiration compared to both other categories and no difference in photoassimilation between TC-44x and TC-22x. These factors, working jointly, contributed to a decreased water-use efficiency of TC-44x leaves. Perhaps this decrease in water-use efficiency, in part, led to decreased carbon fixation of TC-44x plants. A previous study in *Lonicera japonica* reported higher leaf photoassimilation rates in higher ploidy levels when under drought stress, and similar rates under normal water conditions (Li et al., 2009). Perhaps the dynamic of gas exchange readings would shift to favor the TC-44x plants if low-water conditions were applied. Also, temperature was a highly significant (*P* < 0.001) factor for all leaf gas exchange measurements, but no interactions with plant category were observed.

With the ever-increasing influence of governmental regulation of GHG emissions, and the prospect of improving marketability, addressing GHG friendly practices could benefit the ornamental horticulture industry (Marble et al., 2011). In this study, chromosome-doubled *P. laurocerasus* ‘Otto Luyken’ fixed less carbon
than the standard cytotype under the same growing conditions. In terms of an LCA, this result, combined with decreased WUE could show a greater carbon footprint during production. Also, a high percentage of the chromosome-doubled cytotype reverted to lower ploidy-levels, and had an increased vegetative adult phase both complicating nursery production. However, considerations such as reduced pruning of the chromosome doubled cytotype could reduce associated labor and carbon costs. Future work could delve more deeply into overall water use and production differences associated with these plants to better understand the overall effect on carbon budget and production practicality.
Table 5.1. Least squared (LS) means ± LS standard error of weighted whole-plant measurements comparing two independently sourced standard-cytotype ($2n = 22x$) and one chromosome-doubled ($2n = 44x$) cytotype of *Prunus laurocerasus* ‘Otto Luyken’. Measurements presented are from after the first growing season of the study (T1) and after the second growing season of the study (T2).

<table>
<thead>
<tr>
<th>Plant category&lt;sup&gt;y&lt;/sup&gt;</th>
<th>Ploidy</th>
<th>Root: shoot (Ratio)</th>
<th>Water content (%)</th>
<th>Carbon (%)</th>
<th>Nitrogen (%)</th>
<th>Phosphorus (%)</th>
<th>Potassium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1-22x</td>
<td>$2n = 22x$</td>
<td>0.91 ± 0.11 a&lt;sup&gt;y&lt;/sup&gt;</td>
<td>54.6 ± 1.1 a</td>
<td>46.1 ± 0.35 a</td>
<td>1.29 ± 0.17 b</td>
<td>0.25 ± 0.023 a</td>
<td>0.62 ± 0.094 a</td>
</tr>
<tr>
<td>TC-22x</td>
<td>$2n = 22x$</td>
<td>0.95 ± 0.11 a</td>
<td>56.8 ± 1.1 a</td>
<td>45.7 ± 0.35 a</td>
<td>1.40 ± 0.17 ab</td>
<td>0.28 ± 0.023 a</td>
<td>0.68 ± 0.094 a</td>
</tr>
<tr>
<td>TC-44x</td>
<td>$2n = 44x$</td>
<td>0.93 ± 0.13 a</td>
<td>57.0 ± 1.4 a</td>
<td>46.7 ± 0.43 a</td>
<td>1.95 ± 0.21 a</td>
<td>0.31 ± 0.028 a</td>
<td>0.74 ± 0.12 a</td>
</tr>
</tbody>
</table>

<sup>z</sup>Identifies the source of material; N1-22x = nursery sourced standard cytotype, TC-22x = Tissue-culture sourced standard cytotype, TC-44x = Tissue-culture sourced chromosome-doubled cytotype.

<sup>y</sup>Letters represent significant differences ($P < 0.05$) in LS means using an unadjusted means comparison test on N1-22x (n=6), TC-22x (n=6) and TC-44x (n=4).
Table 5.2. Stomatal and portable photosynthesis system (LI-6400XT; LI-COR Inc., Lincoln, NE) measurements comparing two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of *Prunus laurocerasus* ‘Otto Luyken’.

<table>
<thead>
<tr>
<th>Plant category</th>
<th>Ploidy</th>
<th>Stomatal density (no. / mm²)</th>
<th>Guard-cell length (µm)</th>
<th>Photoassimilation (µmol·m⁻²·s⁻¹)</th>
<th>Stomatal conductance (mol·m⁻²·s⁻¹)</th>
<th>Transpiration (mmol·m⁻²·s⁻¹)</th>
<th>Water-use efficiency (mmol·mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1-22x</td>
<td>2n = 22x</td>
<td>141 ± 6.2 a</td>
<td>25.0 ± 0.58 c</td>
<td>10.1 ± 0.26 b</td>
<td>0.165 ± 0.005 c</td>
<td>3.74 ± 0.09 b</td>
<td>2.71 ± 0.033 a</td>
</tr>
<tr>
<td>TC-22x</td>
<td>2n = 22x</td>
<td>138 ± 5.2 a</td>
<td>26.5 ± 0.23 b</td>
<td>11.2 ± 0.49 a</td>
<td>0.186 ± 0.009 b</td>
<td>4.10 ± 0.16 b</td>
<td>2.75 ± 0.061 a</td>
</tr>
<tr>
<td>TC-44x</td>
<td>2n = 44x</td>
<td>66.0 ± 3.5 b</td>
<td>35.8 ± 0.31 a</td>
<td>11.7 ± 0.63 a</td>
<td>0.219 ± 0.012 a</td>
<td>4.69 ± 0.21 a</td>
<td>2.50 ± 0.078 b</td>
</tr>
</tbody>
</table>

Identifies the source of material; N1-22x = nursery sourced standard cytotype, TC-22x = tissue-culture sourced standard cytotype, TC-44x = tissue-culture sourced chromosome-doubled cytotype.

Means ± standard error of the mean on N1-22x (n=3), TC-22x (n=3) and TC-44x (n=3).

Least squared (LS) means ± LS standard error on N1-22x (n=17), TC-22x (n=5) and TC-44x (n=3). Measurements represent an average of individual leaves not whole-plant.

Letters represent significant differences (P < 0.05) in means using Fisher’s protected LSD test.

Letters represent significant differences (P < 0.05) in LS means using an unadjusted means comparison test.
Fig. 5.1. Width, height, (A) and volume (B) measurements comparing three independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of Prunus laurocerasus ‘Otto Luyken’. The sources of material are identified as; TC-44x = tissue-culture sourced chromosome-doubled cytotype, TC-22x = tissue-culture sourced standard cytotype, N1-22x = 3.8-liter nursery-sourced standard cytotype, N3-22x = 11.4-liter nursery-sourced standard cytotype. Time of measurements are identified as: T0 = May 2015, T1 = December 2015, T2 = November 2016. Sample sizes are as follows; TC-44x at T0 (n=23), TC-22x at T0 (n=11), N1-22x at T0 (n=23), N3-22x at T0 (n=5), TC-44x at T1(n=6), TC-22x at T1 (n=8), N1-22x at T1 (n=20), TC-44x at T2 (n=3), TC-22x at T2 (n=5), N1-22x at T2 (n=17).
Fig. 5.2. Photographs of plants before the T1 (December 2015) destructive harvest. Sources of plant material are as follows; TC-44x = tissue-culture sourced chromosome-doubled \((2n = 44x)\) cytotype (A), TC-22x = tissue-culture sourced standard \((2n = 22x)\) cytotype, and N1-22x = nursery sourced standard \((2n = 22x)\) cytotype. The rulers in the photos are 30.5 cm.
Fig. 5.3. Material breakdown of two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of Prunus laurocerasus ‘Otto Luyken’. Material presented from after the first growing season of the study (T1) and after the second growing season of the study (T2). The sources of material are as follows; TC-44x = tissue-culture sourced chromosome-doubled cytotype (n=4) (A), TC-22x = tissue-culture sourced standard cytotype (n=6) (B), and N1-22x = 3.8-liter nursery-sourced standard cytotype (n=6) (C).
Fig. 5.4. Linear regression and best-fit line of total biomass by conical volume using two *Prunus laurocerasus* ‘Otto Luyken’ cytotypes grown in 26.5-liter containers.
Fig. 5.5. Estimated annual carbon fixation of two independently sourced standard-cytotype \((2n = 22x)\) and one chromosome-doubled \((2n = 44x)\) cytotype of *Prunus laurocerasus* ‘Otto Luyken’. The sources of material are as follows; TC-44x = tissue-culture sourced chromosome-doubled cytotype, TC-22x = tissue-culture sourced standard cytotype, and N1-22x = 3.8-liter nursery-sourced standard cytotype. Least squared means presented are back-transformed from a square-root transformation, and standard error (SE) bars represent ± arithmetic SE of the untransformed means. Letters represent significant differences \((P < 0.05)\) in medians using Tukey-Kramer adjusted means comparison test. Sample sizes are as follows; TC-44x at T1 (n=6), TC-22x at T1 (n=8), N1-22x at T1 (n=20), TC-44x at T2 (n=3), TC-22x at T2 (n=5), N1-22x at T2 (n=17).
Fig. 5.6. Stomatal comparison between two independently sourced standard-cytotype (2n = 22x) and one chromosome-doubled (2n = 44x) cytotype of *Prunus laurocerasus* ‘Otto Luyken’. The sources of material are as follows; TC-44x = tissue-culture sourced chromosome-doubled cytotype (A), TC-22x = tissue-culture sourced standard cytotype (B), and N1-22x = 3.8-liter nursery-sourced standard cytotype (C).
Literature Cited


Li, W. L., G.P. Berlyn, and P.M.S. Ashton. 1996. Polyploids and their structural and


General Conclusion

This research was performed to address important themes that have been communicated to us through contact with the vast network of Oregon nurseries. We hope that this research has provided insight, and applicable knowledge to the field of ornamental horticulture as it relates to cherrylaurel production and breeding. Polyploidy in plant breeding stands out as the unifying theme, of which all chapters strive to address.

With the end goal of interspecific hybridization with *Prunus laurocerasus*, *P. lusitanica* underwent successful chromosome doubling procedures (Contreras and Meneghelli, 2016). We anticipate that this $2n = 16x$ form of *P. lusitanica* may be the bridge we need to introgress shothole disease resistance into the $2n = 22x$ *P. laurocerasus*. This chromosome doubled form of *P. lusitanica* also possessed additional beneficial traits that may prove adventitious to an interspecific cherrylaurel hybrid. Such observed traits were compact form and deep-green foliage. The differences observed in stomatal size and density, and increased leaf thickness, were similar to outcomes seen in other rosaceous species (Kadota and Niimi, 2002; Kermani et al. 2003), and, we hope these traits will improve drought tolerance, compounding this cytotypes usefulness as a breeding parent.

As these plants continues development to maturity, we are simultaneously seeking circuitous routes to interspecific hybridization of these two species. As such, in vitro germination techniques were described as a starting point to develop embryo rescue techniques. Cross-pollinations of these two species have shown scarce but
present seed-set with *P. lusitanica* as the mother. Successful embryo rescue has been performed in other *Prunus* species (Kukharchyk and Kastrickaya, 2006; Lu et al., 2007), and we plan to use the developed media and the cultural practices studied to attempt embryo rescue between these two species in the coming years.

The importance of potential hybridization between these two species does not end at shothole disease resistance. Sterility is another important potential outcome of both ploidy manipulation and interspecific hybridization. In attempts to create sterile forms of the weedy *P. laurocerasus*, two cultivars underwent effective chromosome doubling procedures. As these plants developed we studied the morphological and physiological discrepancies between the cytotypes.

One such study compared ability to successfully root and chromosome doubled cytotype of *P. laurocerasus* ‘Schipkaensis’. Although Sülüşoğlu and Çavuşoğlu. (2009 and 2010) have performed extensive research on the vegetative propagation of *P. laurocerasus*, we encountered no reports which previously compared vegetative propagation of two plant cytotypes of these plants or others. Our study found that the chromosome doubled cytotype of the Schipkaensis cultivar rooted at the same percentage rate, and possessed slightly fewer roots per rooted cutting. These plants have successfully moved along in production with no obvious ill-effect. This study presents a favorable result for the use of ploidy manipulation of this species as it does not inhibit vegetative propagation.

To further understand the effects of polyploidization in cherrylaurels, we also compared morphological and physiological traits of chromosome doubled cytotypes of *P. laurocerasus* ‘Otto Luyken’. The chromosome doubled form of the Otto
Luyken cultivar was smaller in both height and width in most cases, possessed similar nutrient composition under favorable production conditions, assimilated CO$_2$ in the leaves at the same rate, had increased transpiration and stomatal conductance, and decreased water-use efficiency. We concluded that these characteristics, working collectively, resulted in a decreased ability to fix carbon, compared to the standard cytotype. Again, this provides insight to production discrepancies and how such discrepancies may affect the decision making for plant breeding and production of cherrylaurels is affected going forward.

As part of a land grant university, the Ornamental Plant Breeding Program here at Oregon State University is committed to providing continued applicable research on topics that are important to Oregon nurseries, and the nursery industry as a whole. Cherrylaurels are economically important, and nursery professional have expressed room for improvement. We hope that this research has shed light onto the steps we are taking to improve their marketability and production performance, and such insights may expand the base of knowledge as we move cherrylaurce science forward.
Literature Cited


Kehr, A.E. 1996. Polyploids in rhododendron breeding. J. Amer. Rhododendron Soc. 50:4


Marchi, G., T. Cinelli, and G. Surico. 2014. Bacterial leaf spot caused by the quarantine pathogen Xanthomonas arboricola pv. pruni on cherry laurel in central Italy. Plant Dis. 98:1600 (abstr.).


Appendices
Appendix A: Chromosome Doubling of *Prunus lusitanica* Using an Oryzalin Seedling Soak

A chromosome doubling experiment was performed to supplement the experiment performed in chapter 2. We hoped to learn if soaking germinated seedlings in an oryzalin bath could effectively double the chromosomes of the *Prunus lusitanica* (*2n = 8x*). Two concentrations (50 µM and 125 µM) of oryzalin in H₂O with 1% Dimethyl sulfoxide (DMSO) plus a control with 1% DMSO were used to soak seedlings for 24 hours in a shaker moving at 100 rpm. Eighteen seedlings were present in each solution. Seedling survival was 89%, 83% and 89% for 50 µM oryzalin, 125 µM oryzalin, and the control, respectively. We found three mixoploids (*2n = 8x + 16x*) resulting from the 125 µM treatment, and three mixoploids plus one *2n = 16x* resulting from the 50 µM treatment. We also found a putative *2n = 12x* in the control group, which agrees with chapter 2’s discussion on unreduced gametes in this species. This method appears to effectively double the chromosomes of this species with far fewer working hours. However, in future studies using this method and species I would recommend increasing the concentration of oryzalin to get the seedling mortality closer to 80% for less screening of seedlings and increased chromosome doubling potential.
Appendix B: R Code for Kruskal-Wallis Rank Sum Test with Means Comparison and Adjustment

```r
data <- read.csv("PLusSurvival.csv", header=TRUE)
library('car')
library('lsmeans')
library("agricolae")
treat <- data$Treatment
block <- factor(data$Block)
survival <- data$Survival

boxplot(survival ~ treat)
leveneTest(survival ~ treat)
qqnorm(survival)
qqline(survival)

plot(survival~block)
leveneTest(survival~block)
anova(lm(survival~block))

#Does not pass assumption tests
T1 <- kruskal(survival,treat,alpha=0.05,p.adj= "bonferroni", group=TRUE, main = NULL, console = FALSE)
T1

The Kruskal-Wallis rank sum test is a non-parametric test that ranks outcomes then averages the ranks for comparisons. This test does not assume normality or equal variances. Kruskal-Wallis was used for this analysis because data were non-normal and variances were unequal and therefore failed a Levene’s test for homogeneity of variance. Kruskal-Wallis can only be used for a one-way ANOVA so blocks were ignored. Mean comparisons applied with a Bonferonni adjustment to account for family-wise error.
Appendix C: R Code for Logistic Regression

```r
# Load data
d <- read.csv('PLusGreenSeed.csv', header = T)
library(car)
library('agricolae')
require(lmtest)

gm1 <- glm(d$Radicle ~ d$GA3 + d$BAP + d$Surcose, data=d, family=binomial())
gm12 <- glm(d$Radicle ~ d$GA3 + d$Surcose, data=d, family=binomial())
lrtest(gm1,gm12)
gm13 <- glm(d$Radicle ~ d$GA3, data=d, family=binomial())
lrtest(gm12,gm13)
gm14 <- glm(d$Radicle ~ d$Surcose, data=d, family=binomial())
lrtest(gm12,gm14)

gm02<- glm(d$Shoot ~ d$GA3 * d$BAP * d$Surcose, data=d, family=binomial())
gm2 <- glm(d$Shoot ~ d$GA3 + d$BAP + d$Surcose, data=d, family=binomial())
lrtest(gm02,gm2)
gm22 <- glm(d$Shoot ~ d$GA3 + d$Surcose, data=d, family=binomial())
lrtest(gm2,gm22)
gm23 <- glm(d$Shoot ~ d$Surcose, data=d, family=binomial())
lrtest(gm22,gm23)
gm24 <- glm(d$Shoot ~ d$GA3, data=d, family=binomial())
lrtest(gm24,gm22)
```

Since radicle and shoot emergence are a yes/no response it is difficult to quantify responses if not blocked into small groups. Logistic regression assumes a logistic response curve of yes/no responses and estimates average response for a treatment. To test an effect log likelihood tests are used to comparing full to reduced models. The effect of a treatment is seen when the log likelihood test is significant after removing a particular factor from the model. The same steps were taken to test weeks after pollination and cold-stratification for the second part of the experiment.
Appendix D: R Code for Kruskal-Wallis Rank Sum Test

d <- read.csv("skiprooting.csv", header=T)
library(car)

ploidy <- d$Ploidy
year <- d$Year
root <- d$Rooting

kruskal.test(root ~ year)
kruskal.test(root ~ ploidy)

Data for rooting percentage was non-normal and had unequal variance so a non-parametric Kruskal-Wallis rank sum test was used. Since this test must use a one-way analysis of variance (ANOVA) it was performed on ploidy and year separately.
Appendix E: SAS Code for Repeated Measures Analysis

PROC MIXED data=Carbon;
CLASS Time Type Accession;
MODEL srco2= Type Time Type*Time;
RANDOM Accession;
REPEATED Time/ sub=Accession type=un r rcorr;
LSMEANS Type Time/ CL;
LSMEANS Type Time Type * Time/ diff=all;
RUN;

The code above was used to address repeated measures since many of the
same plants were measured at both T1 and T2. The “Repeated” statement identifies
that the same accessions appear in both times. Any effect of an accession is assumed
to be a random effect.

PROC MIXED data=Licor;
TITLE Photosynthesis;
CLASS temp plant source;
MODEL photo=source temp temp*source;
RANDOM Accession;
REPEATED temp /subject=Accession type=un r rcorr;
LSMEANS temp source/pdiff;
RUN;

The code above was once again used to address repeated measures, this time
for use with photosynthesis system measurements. The “Repeated” statement
identifies that the same accessions are measured at different temperatures.