Prunus laurocerasus: A Study of Reducing Gamete Development through Haploidy and Determining Cytotype Variability in Pollen

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James Tyler Seely for the degree of Bachelor of Arts in International Studies in Horticulture presented on November 02, 2018.

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Abstract approved: _____
Ryan Contreras, Ph.D.

Prunus laurocerasus, commonly known as cherrylaurel, is a woody shrub grown in temperate climates for its durability and landscape appeal. Due to its abundance of drupes that are spread by birds, cherrylaurel is a litter nuisance in cultivation and also has escaped cultivation such that it has naturalized. Furthermore, it is a host of western cherry fruitfly, a pest that impacts growers' ability to export plants to neighboring states. For these reasons, a sterile form of cherrylaurel is highly desired and investigating methods to produce such a cultivar was the goal of the current study. Anther culture was attempted to create haploid ($n = 11x = 88$) cherrylaurel plants incapable of forming embryos in developing seed. Additionally, pollen grains were measured from five different ploidies to determine how pollen size changes with increased ploidy level and also identify ploidy level of the germ layer in cytochimeras. The results of our anther culture experiment were inconclusive. Callus was developed on several samples, though callus began to die after five weeks. In future experiments sucrose concentrations will be reduced and callus will be moved to fluorescent lighting after 30 days to mimic other studies in the genus. Pollen measurements identified three statistically significant groups. Standard cytotype ($22x$) plants had smallest pollen, $33x$ and cytochimeras were larger but not different from each other, and $44x$ plants had the largest pollen. Our results show that pollen size does increase with ploidy, and that all accessions observed still show pollen production.

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Bachelor of Arts in International Studies in Horticulture thesis of James Tyler Seely presented on November 02, 2018.

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I understand that my thesis will become part of the collection of Oregon State University. My signature below authorizes release of my thesis to any reader upon request. I also affirm that the work represented in this thesis is my own work.

_____

James Tyler Seely, Author

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Chapter 1: ANTHER CULTURE OF *PRUNUS LAUROCERASUS* TO PRODUCE HAPLOID PLANTS

Introduction

Prunus, a genus within the Rosaceae family, is wide ranging and can be found occupying a variety of cultivated landscapes from the agricultural to maintained ornamental settings. *Prunus laurocerasus*, a common ornamental species, has a habitat that ranges from USDA Hardiness Zones 6-8 (Dirr, 2009; USDA, 2018). *Prunus laurocerasus* is a spreading evergreen shrub. In its natural habitat, plants may become tree-like, though cultivated forms become more bush-shaped (Missouri Botanical Garden, 2018). Often called cherry laurel or English laurel, *Prunus laurocerasus* is a popular landscape plant, enjoyed for its ability to create tight hedges, and does well in shaded areas by itself or in large groups of up to 100 (Dirr, 2009).

Prunus laurocerasus has a wide native range, which stretches from Bulgaria and Yugoslavia in southwestern Europe, over to northern Iran, Turkey, Armenia, Azerbaijan, Georgia, and southern Russia in Asia (Queensland Government, 2016). Many cultivated species of *Prunus* related to cherry laurel, such as *Prunus avium* (sweet cherry) and *Prunus cerasus* (sour cherry), have also emerged from a similar geographical region (Marti et al., 2012). *Prunus laurocerasus* is not to be confused with two related ornamental species, *Prunus caroliniana* and *Prunus lusitanica*, which are both common landscape plants. The former (*P. caroliniana*) is native to the North American southeast, while the latter (*P. lusitanica*) has a native habitat in Portugal and Spain (North Carolina State University, 2018; Oregon State University, 2018). While *Prunus laurocerasus* does not appear to be problematic within its native range, regions

with similar climates around the globe have struggled with their invasion. *Prunus laurocerasus* has become naturalized in many parts of the world including places as isolated geographically as Australia and Tasmania (Queensland Government, 2016).

Records indicate that *Prunus laurocerasus* has been used as an ornamental landscape plant in Europe as early as the mid 16th century when a traveled established several plants in his garden in Genoa, Italy. *Prunus laurocerasus* became further established in the European ornamental market in 1576 when the Flemish botanist Carolus Clusius (1526-1609), who also popularized the tulip, brought it to the United Kingdom. Clusius, who discovered the half-dead specimen coming from his correspondent in Constantinople, David Ungnad, nurtured the plant back to life. For the first half of the 17th century, cherry laurel was thought to be too tender to be grown outdoors in England. However, by the middle of the 17th century with the popularization of the naturalistic espalier style of Capability Brown (1716-1783), *Prunus laurocerasus* was planted close together and popularized as a hedge as it can be found today (Klingaman, 2009).

In the Pacific Northwest of the United States, *Rhagoletis indifferens* (western cherry fruit fly), a native Diptera of North America, is a key pest to *Prunus avium* (sweet cherry). Reports of cherry damage due to *Rhagoletis indifferens* in the Pacific Northwest emerged around 1900, and was later found in the Yakima Valley in 1942. The fruit fly has been known to develop in the ripening commercial and wild crops of *Prunus avium*, and *P. cerasus*. If larva develops in ripening cherries and uncontrolled, larvae can ruin the majority of fruit in an orchard. Since adults are weak migrators, much of the pest will stay within an orchard and could seriously affect the marketability of the cherry fruit crop (Messina and Smith, 2010).

Spotted wing Drosophila, *Drosophila suzukii*, native to eastern and southeastern Asia, is another large pest of cherry and other soft fruits. *Drosophila suzukii* was first detected in 2008 in

California, and has since spread to Oregon and Washington to become major pests of soft fruits, affecting: cherry, raspberry, blackberry, blueberry, strawberry, peach, plums, pluots, nectarines, juice grapes, table grapes, and wine grape (Walsh et al., 2011). *Drosophila suzukii* has obtained worldwide presence due to the global plant trade and international fruit market. According to the EPPO Global Database, as of July 25th, 2018, *Drosophila suzukii* had been spotted in 48 countries (European and Mediterranean Plant Protection Organization, 2018). Evaluations of the economic impacts of *Drosophila suzukii* have indicated that revenue losses exceeded 500 million U.S. dollars in the western states of Oregon, California, and Washington (Bolda et al., 2010).

Research published by the Entomological Society of America and Oregon State University reported that *Prunus laurocerasus* will harbor both *Rhagoletis indifferens* and *Drosophila suzukii* (Lee et al., 2013; Yee and Goughnour, 2005). *Prunus laurocerasus*, being a common landscape plant and being able to harbor these destructive pests, contributes to the increase and distribution of these fruit fly populations. Since much of Oregon, Washington, and California rely heavily on the production of soft fruits, the ability to make a sterile *Prunus laurocerasus* selection could potentially cut down on pest pressure in orchards. Additionally, with major fruit production occurring around the world, a sterile *Prunus laurocerasus* selection would allow for the reduction of risk of fruitfly and plant invasion to their non-native regions. We could not find any historical research in our investigation that mentioned procedures in place for limiting these fruit fly populations directly in *Prunus laurocerasus*. This is likely because of the sheer population size of *Prunus laurocerasus* as an ornamental landscape plant in temperate regions, and because the fruit is non-essential to the plant's appeal in an ornamental landscape.

Prunus laurocerasus, whose wild-type chromosome count of $2n = 22x = 176$, is the highest in the genus and is curiously high for woody plants. Common cultivars of this species

that are used in landscape design are ‘Otto Luyken’, ‘Zabeliana’, ‘Mt. Vernon’, and ‘Marbled Dragon’ (Oregon State University, n.d.). In more recent research, the Contreras Lab has created populations of 33*x* plants to evaluate their ploidy's influence on sterility, though plants are still under evaluation (Contreras and Meneghelli, 2016).

Sterility is often characteristic of odd numbered ploidy levels. In the case of triploids (3*x*), while they are characteristically sterile, there is the possibility that sterility will not be complete. Sterility in odd-numbered polyploids, like that of triploids, lies in the incomplete paring at the time of meiosis. Two pairing possibilities for triploids exist and consist of the creation of either a trivalent or a bivalent plus a univalent. If a bivalent plus a univalent is produced, it is likely that the bivalent pair will be polarized to one side during meiosis while the univalent will be polarized to the opposite side. If the sporophyte contains an odd numbered ploidy or has a complicated genome structure (i.e. aneuploidy or interspecific hybrid), sterility may occur. With the problem of genome imbalance, the effect is almost always deleterious due to the proportions of the genes in the extra chromosomes. This effect, often expressed at the level of gametes, often makes them nonfunctional, though functionality could still be possible. At the zygotic level, lethality, sterility, or lowered fitness can result from these changes (Griffiths et al., 2000). While sterility is often the goal of the creation of odd-numbered polyploid plants, lowered fitness is a possibility, and plants with a lowered fitness could still produce viable pollen. 33*x P. laurocerasus* plants therefore, as seen with the incomplete sterility phenomenon in triploids, may still produce viable pollen.

The creation of haploid 11*x* accessions of *P. laurocerasus* may be a reliable option in creating sterile plants. Because of the substantial chromosome number of *P. laurocerasus* however, pollen production is still a possibility in the haploid state. However, cutting

chromosome numbers in half could reduce pollen to levels where pollination rarely occurs or complete sterility is created.

The purpose of this research is to identify whether anther culture can be used to create a haploid selection of *Prunus laurocerasus*, the goal of which would be to create a sterile, non-fruiting selection in order to avoid harboring destructive species of fruit fly and avoid the plants escape out of cultivation. Additionally, by creating a haploid plant, we hope to reduce the probability of reversion to its wild-type ploidy. The hopeful result of a haploid plant will be complete sterility and the ability to control plant invasiveness and the harboring of destructive species of fruit fly in the fruit. While this research is important to our stone fruit and berry production in the Pacific Northwest, our research also applies globally as we try and reduce the spread of plant and insect species. With the popularity of *Prunus laurocerasus* in many temperate countries, solutions to issues of invasion and escape from cultivation are necessary in order to promote healthy agricultural and landscape systems.

Materials and Methods

Preparation of* P. laurocerasus *inflorescence buds for meiotic analysis

Prunus laurocerasus 'Otto Luyken' inflorescence buds were collected on February 2nd, 2018 from a specimen on the Corvallis campus of Oregon State University immediately north of the Agricultural and Life Sciences Building at 0800HR. Immature 10 - 15mm buds were selected and removed in their entirety from the bush and placed into a beaker for transportation back to the lab.

Carnoy's solution (6 ethanol: 3 chloroform: 1 glacial acetic acid) was mixed to aid in the chemical preservation of the *P. laurocerasus* buds. Freshly collected *P. laurocerasus* buds were

submerged in Carnoy's solution and then vacuum infiltrated for 20 mins. After 20 mins the vacuum was turned off and the buds were incubated at room temperature in Carnoy's solution for 24 h. Following the 24 h incubation, the Carnoy's solution was drained off completely and replaced by 100 ml of 70% ethanol before being set aside at room temperature for 48 h. After completion of the 48 h wait time, the *P. laurocerasus* buds in solution were put under refrigeration at 4 ˚C for storage.

At time of evaluations, buds around 13 mm in length, representing the average between 10 and 15 mm, were removed from the 70% ethanol solution and placed on a clean microscope slide. Beginning at the base of the inflorescence, bud scales were peeled back to expose individual florets. Selected immature florets within buds were removed from the base of the inflorescence and set on the clean microscope slide with a drop of 70 percent ethanol. Immature anthers were removed from the flower buds with fine forceps and macerated in a drop of 2% acetocarmine. After 5 s, a glass coverslip was placed over the macerated and stained anthers and put under the compound microscope (Axio Imager A1; Zeiss Microscopy, Oberkochen, Germany) to observe microspore development. The uninucleate state was the targeted cellular stage for proceeding to anther culture in vitro.

Uninucleate staged microspore cells appeared to be prevalent in the sample (Figure 1). No fully developed pollen grains were observed at this time.

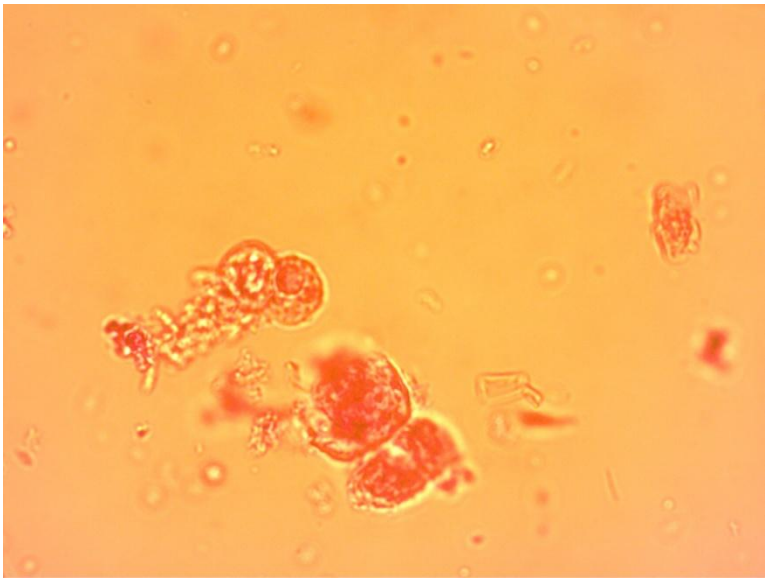


Fig. 1. Microspore cells of 'Otto Luyken' cherrylaurel in uninucleate stage.

Anther culture of P. laurocerasus *buds*

Several small inflorescence buds were collected from *Prunus laurocerasus* 'Otto Luyken' on the Corvallis campus of Oregon State University immediately north of the Agricultural and Life Sciences Building at 0800HR on February 8th, 2018 and brought back to the lab in a plastic beaker. Back in the lab, three buds at a length of approximately 13 mm were selected because they corresponded to the lengths of the buds checked for microspores. Excess buds were put into tap water and stored in the refrigerator. One inflorescence bud that was frozen in ice for 12 hours was also used for this experiment and followed the same procedure as all other samples following this treatment.

Selected buds were put into a tea steeper to be surface sterilized with 70 percent ethanol for 30 seconds and a 5% bleach solution for 10 minutes. After the surface sterilization process buds were rinsed with sterile water three times and then put on a sterile petri dish in the laminar flow hood. Individual florets were then excised from the inflorescence. Florets from the bottom and top halves of an inflorescence were collected separately to assess whether more immature

flowers on the inflorescence would have different results. Each collection of florets was placed in its own respective 1.5 ml microfuge tube containing 1ml of 4 ˚C B5-13 wash (Sigma Aldrich, St. Louis, MO) with modifications. Our B5 media sucrose level was adjusted to 13% by weight and had no added agar. Florets in the media were then gently macerated with fine-tipped tweezers. At this point two microfuge tubes contained the bottom halves of each of the two buds (labeled B) and two microfuge tubes contained the top halves of the flower buds (labeled T). Cultures from frozen bud were labeled FB and FT for "frozen bottom" and "frozen top" and kept separate from unfrozen material. Microfuge tubes were refrigerated for 1.5 h then the solution with macerated floral material was strained through sterile tea strainers into new tubes. New tubes were capped, labeled, and then placed into a centrifuge for two minutes at 2000 rpm. Tubes were removed from the centrifuge and surface sterilized with 70% ethanol to prevent contamination. Supernatant was removed using a 1 ml micropipette set to 0.5 ml, preserving the pellet. Fresh B5-13 (750 µL) was added back to the tubes and the tubes were centrifuged again for two minutes. Upon removing the tubes they were sterilized again with 70% ethanol. Process was repeated twice more for a total of three B5-13 washes. After final centrifugation, pellets were resuspended in 1.5 ml of room temperature NLN medium (*Phyto*Technology, Shawnee Mission, KS). Each centrifuge tube was emptied onto a separate 60 x 15 mm perti dish containing 2 ml of room temperature NLN media (3.5 ml total/plate). Plates were wrapped in parafilm to decrease evaporation and prevent contamination and labeled with initials of the technician, date of transfer, and T or B to mark the contents original position on the inflorescence buds. Petri dishes were loaded into a non-shaking, dark incubator at 25 ˚C for 11 days and then moved on day 12 to a dark, shaking incubator at 25 ˚C where they remained for the rest of the experiment.

Each week samples were taken out of the incubator and observed under the microscope for callus formation or changes in phenotype. Stereo microscope was set to a standard of 10x to observe the calli over the experiment.

Plates with callus growth after 47 days were transferred to a semi-solid NLN media (PhytoTechnology, Shawnee Mission, KS) with modifications, using 1.77 grams potato dextrose agar with 3% sucrose. 0.1 mg/l of IAA and 0.05 mg/l of BA was also added to the semi-solid media after autoclaving. Samples transferred to semi-solid media were placed back in the dark 25 ˚C incubator on a non-shaking shelf and observed weekly.

Results

Both frozen and unfrozen trials produced growth with the standard non-freezing trial only producing results with the base florets, and the freezing trial producing growth on both the top and bottom florets (Table 1). By the end of the experiment, plates 1B, 2B, 1BF and 1TF had produced callus, consisting of both compact and friable types.

Weeks one and two saw the greatest change in the experiment with the development of calli. Weeks three and four saw marginal increases in growth but calli health remained stable. Week five and six saw a decrease in calli health in plates 1BF and 1TF as calli began to turn brown and die. Plate 1BF saw a quicker browning than plate 1TF, and by day 32, one of two calli were dead. A strange yellow contamination, thought to be duplicating floral tissues, was found in plate 1BF during week two. The contamination continued to spread throughout the culture and by day 47, had killed both calli in the liquid culture.

Following the move from the 13% sucrose liquid NLN to the 3% sucrose solid NLN media, calli health slowly declined in all of the plates.

Interestingly, callus formation differed between the calli with some having cells that were tightly packed together and compact, and others being friable, loosely packed callus. Plate 2B, while on the liquid NLN media for example, contained callus where cells were tightly packed together (Fig. 2 (a)), and callus where cells were loosely packed (Fig. 2(b)). One callus on plate 2B also showed strange growth with the presence of a bright red coloring in some of the cells (Fig. 2(b)).

No observable growth was noticed after the transfer to the solid NLN media. Visual observations were continually collected on callus until May 3rd, 2018, 12 weeks after initial culture in liquid media and five weeks following their transfer to solid media. All calli were presumed dead following May 3rd, 2018 because no growth had been observed in five weeks and all calli were brown, suggesting that they were no longer living.

Discussion

Flow cytometry was originally the plan for identifying the ploidy of the callus that was produced in this experiment, however, the experiment did not produce large enough calli for analysis. Additionally, calli showed signs of death before gaining enough volume to be analyzed, and due to cellular breakdown following death, it is not likely that the browning calli would have been able to be analyzed for ploidy. As such, this experiment showed that calli can be produced using the explained methods, though media composition should be modified in order to optimize callus growth and stability.

Likely, it is sugar composition that may need to be modified for further success in this experiment. Although growth results were seen with a higher sugar content (13%) and death of calli was seen in lower sugar content (3%), beginning with a sugar content around 5% could show better results. Similar anther culture research done with *Prunus dulcis* shows promising

results with sugars around 6% (Cimò et al., 2017). Maintaining a total sugar content at or below 6% should hopefully provide similar results to the *Prunus dulcis* study.

During our experiment, callus was kept in the dark for the whole experimental period, and this could have been problematic for the later growth of the calli produced. The *Prunus dulcis* study transferred the anthers in culture to a cool, fluorescent light after 30 days. Following the change, they saw further development in the anthers and calli. In further experiments of our own with *Prunus laurocerasus*, moving the newly initiated calli to fluorescent light might prevent the calli from turning brown.

Unfortunately, the study with *Prunus dulcis* was found after our experiment was completed, so reducing the sugar and adding fluorescent lighting after 30 days may contribute to greater success in our future experimentation.

Because there were only two samples of the frozen trial, it cannot be said if it was an anomaly that 100% were successful, or because the treatment had some physiological influence that increased success. However, future trials using a light freezing method would be helpful in understanding whether increased success can be found. In the *Prunus dulcis* study found after our experiment was completed, one week of bud cold treatment at 4 ˚C was provided before florets and anthers were excised. This cold treatment, followed by their heat treatment at 35 ˚C for one week, likely shocked the cells and contributed to a physiological change allowing the cell to divide undifferentially into callus. The light-freezing treatment in our experiment could have provided a similar, yet more instantaneous affect. More experiments will be needed to test this hypothesis.

The results of the standard non-freezing trial also show promise for future success. While the top florets used did not provide any callus growth, florets from the bottom of the

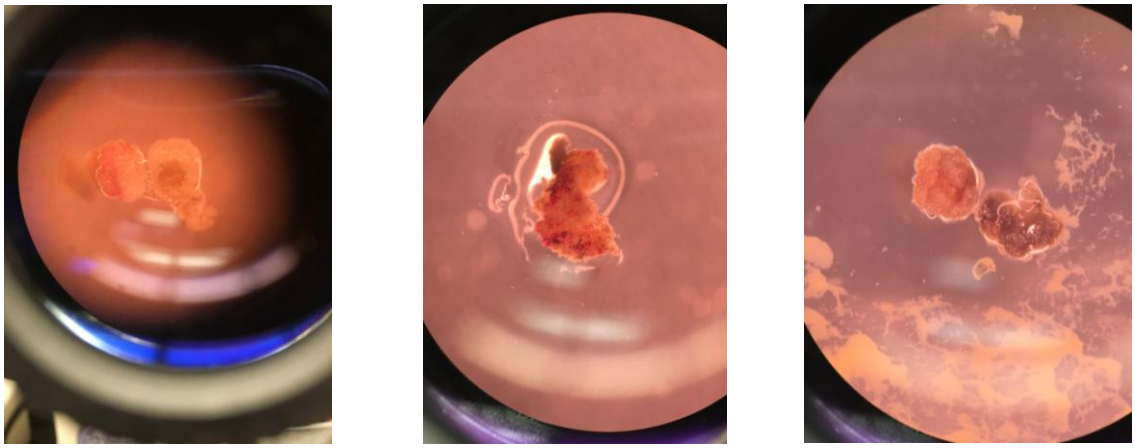
inflorescence were able to provide cells capable of dividing into callus. The reason for which the top florets did not promote callus growth eludes, but an over-successful attempt in filtering pre-treatment may have eliminated or killed all viable cells. More replications in future experiments will allow us to see if florets from the upper 50% of the inflorescence are able to produce callus as well. An additional hypothesis to why these portions did not promote callus growth is that these portions of the inflorescence, having less developed florets, may have not had microspores developed enough to provide for callus growth.

The implications of this study can be applied internationally as *Prunus laurocerasus* is a very widespread species in both the United States and Europe. Improving on anther culture methods could result in producing our goal of haploid plants and reducing this species' ability to naturalize in other temperate regions. Additionally, reducing chromosome levels and creating fruitless cultivars will in turn contribute to the degradation of habitat for both *Rhagoletis indifferens* and *Drosophila suzukii*, protecting cultivation of soft fruits in production.

Table 1. Callus production on plates

Plate number	Callus? Yes/No
1B	Yes
1T	No
2B	Yes
2T	No
3B	No
3T	No
4B	No
4T	No
1BF	Yes
1TF	Yes

Key:
T = Top of inflorescence
B = Bottom of inflorescence
F = Inflorescence was lightly frozen before being cultured



(a) (b) (c)

Fig. 2. Callus production of *P. laurocerasus*. (a)Plate 2B shows callus 12 days post culture in liquid NLN media while (b) shows callus of the same plate (2B) nine weeks after original culture in solid NLN media. Red coloration and a friable cellular structure has appeared. (c) Shows callus of freezing trial plate 1BF in liquid NLN 32 days post culture.

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Chapter 2: POLLEN SIZE IN RELATION TO PLOIDY

Introduction

Ploidy level is known to be associated with cell volume in plants. Literature shows that it is common for polyploid organisms to develop larger bodies due to an increase in cell size (Vining et al., 2016). Using this knowledge, pollen can be observed to identify whether increases in ploidy result in larger pollen sizes. With over 45 cultivars currently in the ornamental plant market, *Prunus laurocerasus* is an autopolyploid with a chromosome complement of $2n = 22x = 176$. The high chromosome count of *Prunus laurocerasus,* which is the highest in the *Prunus* genus and unusually high for woody plants, may contribute to irregularities during meiosis.

Prunus laurocerasus produces large drupes which are often a nuisance in landscapes when they drop onto sidewalks. In addition, *Prunus laurocerasus* has escaped cultivation in northwest regions of the United States, as well as other temperate regions of the world, and raises questions about its potential to spread (Contreras and Meneghelli, 2016). Historically, to overcome the problem of fertility, many breeding programs produce triploid plants to induce seedlessness or another form of sterility. Interestingly, in the case of the drupe, immature fruit will drop should the embryo not develop (Racskó et al., 2007). In the case of *Prunus laurocerasus,* disrupting embryo development will not only reduce invasion of fruit fly larvae in the fruit, but will also reduce the plant's invasiveness in the environment as birds will not be able to carry and distribute seed.

In this study, we observed difference in pollen sizes based on ploidy. Several cytotypes including $22x$, $22x44x$, $33x$, $33x44x$, and $44x$ plants have been produced by the Contreras Lab at Oregon State University, and each will be evaluated to see if increasing diameter of pollen grains

can be correlated to increases in ploidy. Our hypothesis is that there will be a correlation between increasing ploidy and pollen size. As described in the Vining et al. study, larger plant organs can be observed with increasing ploidy, so we hypothesize that this will be the case for pollen. Along with determining how average pollen size changes with ploidy, we also aim to determine which histogenic layer of mixaploid plants are polyploid, and which are 22*x*.

Materials and methods

The pollen of 24 *Prunus laurocerasus* plants were collected for this experiment. The plants were located in the poly high-tunnel at the Lewis-Brown Horticulture Farm on Peoria Road in Corvallis, Oregon. Planted in #5 containers, the 24 plants represented five ploidy levels including 22*x*, 22*x*44*x*, 33*x*, 33*x*4*x*, and 44*x*. Anthers were collected using forceps and set in100mm x15mm petri dishes. The anthers from 1-2 inflorescences were collected on April 20th, 23rd, and 27th. After collection, petri dishes with anthers were taken back to campus and left open for 24 hours to dry over Drierite at room temperature. After the 24 hours, petri dishes were closed back up and stored in a closed container with Drierite in the refrigerator at 4 ˚C until observation.

At time of measurement pollen was taken out of the refrigerator and brought to room temperature before staining. Using a small paint brush cleaned in 70% ethanol and dried, pollen was collected and deposited onto a clean microscope slide. Pollen was suspended in 100 μL of 2% acetocarmine and let stand for 10 seconds before applying the cover slip. An attempted amount of 100 pollen grains per accession was measured under the microscope in micrometers.

Pollen size was observed using a magnification of x100 under a compound light microscope (Axio Imager A1; Zeiss Microscopy, Oberkochen, Germany) with an attached

camera imager (AxiaCam MRm; Zeiss) for viewing on the connected computer monitor. After focusing, a variety of images were taken with multiple pollen grains in order to ensure that at least 100 grains were represented. Following this procedure, the pollen grains in the pictures were measured in micrometers using image analysis software (AxioVision; Zeiss).

Results

All accessions used in this experiment produced stained pollen, however, no single accession can be defined as sterile using this data because pollen germination experiments were not done. All samples contained ample amounts of pollen grains to grant mechanical movement from one inflorescence to another. 33*x* plants were thought to be promising at producing no pollen, but this experiment proved that *P. laurocerasus* is an exception. While some odd-numbered ploidy levels in plants can be found to be sterile in other species, 33*x P. laurocerasus* produced substantial amounts of pollen in some accessions and small amounts of pollen in others. All ploidy levels have the potential to produce large or small amounts of pollen, just as the 33*x* accessions did.

According to ANOVA analysis, ploidy level does have a significant effect on pollen size ($P<0.001$). Tukey HSD results determine with 95% confidence that plants with 44*x* have pollen that is significantly different in size from 22*x* (Table 2, Figure 5). 44*x* plants have an average of 52.48 μm while 22*x* plants have an average of 43.79 μm, making them easily distinguishable from one another. While 22*x* plants contain pollen that is significantly different in size than the pollen of 44*x* plants, pollen from the 22*x*44*x*, 33*x*, and 33*x*44*x* plants have similar averages at 50.06 μm, 51.39 μm, and 49.07 μm respectively.

In the present study, pollen sizes were observed to be relative to ploidy level. Traditionally, pollen size is thought to be positive to ploidy. In our research, 44*x* pollen showed a

significantly larger pollen than 22*x*. Altogether, three statistically different plant groups were determined based on pollen diameter. For mix-ploidy accessions, the pollen size is not a determining factor in allowing ploidy to be determined. Pollen sizes of 22*x*44*x* and 33*x*44*x* showed little to no difference between one another and were not significant based on size. The difference in average pollen size between 33*x* and 44*x* plants was not significant either. Pollen from the 22*x*44*x* accessions showed the widest distribution range among all other ploidy, likely the result of both the 22*x* and 44*x* tissues together (Figure 7).

Pollen size variations of even numbered ploidies are smaller than off number ploidies and mixed ploidies. Theoretically, even ploidies, in our case 22*x* and 44*x*, undergo less problems in meiosis than odd numbered ploidies. Although all the accessions in our research show staining, pollen from even numbered ploidy show more uniformity. This result might imply that the pollen synthesis process is more stable in even numbered accessions, however, more research needs to be done to prove this theory.

Pollen density might further impact the fertility of each accession. While all cytotypes produced pollen, it was noticeable that some of the 33*x* and 33*x*44*x* cytotypes contained less pollen per unit area than did non 33*x* cytotypes (Figure 6). While not all accessions that contained 33*x* material had reduced pollen numbers, enough plants showed the pattern to warrant suspicion about the reduced fertility of these accessions.

Plants within each ploidy were also compared to other plants within their same ploidy. Results show that all ploidy groups except 22*x* contain multiple significant groups.

Table 2. Mean pollen diameter of cherrylaurel plants at various ploidy levels.

Ploidy	n	Average diameter (µm)
22*x*	3	43.8c[z]
22*x*44*x*	10	50.1b
33*x*	6	51.4a
33*x*44*x*	2	49.1b
44*x*	3	52.5a

[z]Values within columns followed by different letters are statistically different based on Tukey's HSD with a 95% confidence interval.

Table 3. Mean pollen diameter within 22*x* accessions

Accession #	Average pollen diameter (µm)
15_0015_07	43.66a[z]
15_0015_16	43.55a

[z]Values within columns followed by different letters have no significant difference based on T-test results.

Table 4. Mean pollen diameter within 22*x*44*x* accessions

Accession #	Average pollen diameter (µm)
12_2	55.65a[z]
4_1_3	55.60a
19_4_2	53.40a
17_2_2	53.05a
13_1_3	48.92b
5_6	47.68bc
15_2_4	47.42bc
5_2	47.29bc
16_2_2	46.03bc
5_3_1	45.18c

[z]Values within columns followed by different letters are statistically different based on Tukey's HSD with a 95% confidence interval.

Table 5. Mean pollen diameter within 33*x* accessions

Accession #	Average pollen diameter (µm)
19_2_1	61.04a[z]
4_1_1	53.72b
20_2_2	52.82bc
20_3_2	50.48cd
19_2_3_2	49.52d
5_4	48.36d

[z]Values within columns followed by different letters are statistically different based on Tukey's HSD with a 95% confidence interval.

Table 6. Mean pollen diameter within 33*x*44*x* accessions

Accession #	Average pollen diameter (µm)
1_3_2	51.36a[z]
3_1	46.25b

[z]Values within columns followed by different letters are statistically different based on Tukey's HSD with a 95% confidence interval.

Table 7. Mean pollen diameter within 44*x* accessions

Accession #	Average pollen diameter (µm)
18_3_2	54.33a[z]
13_2_6	52.20ab
1_3	51.27b

[z]Values within columns followed by different letters are statistically different based on Tukey's HSD with a 95% confidence interval.

Discussion

The results of our experiment provided three distinct groups of significant averages for the five different cytotypes in this experiment (Table 2). Our results were surprising because we had expected 22*x*, 33*x*, and 44*x* plants to have significantly different average pollen sizes, though our Tukey HSD result suggested that 33*x* and 44*x* differences in average pollen size were not significant. Additionally, the difference between 33*x* and 44*x* cytotypes with 22*x* cytotype was significant with a difference around 20%.

Mixaploid plants were another curious investigation as we were unaware which histogenic layers contained the chimeric tissue. Following our experiment, we can say with confidence that the L-II histogenic layer of some of the 22*x*44*x* plants likely contain both 22*x* and 44*x* tissues. With the L-II histogenic layer contributing to the development of gamete cells in the plants, this is one reason to explain why the average size of pollen of the 22*x*44*x* plants are distributed over a broader range than other cytotypes. With both cytotypes forming gametes in the 22*x*44*x* plants, both 22*x* and 44*x* pollen is produced. Since the average pollen size of both cytotypes is significantly different, averaging about 8.7 µm, this explains the wide distribution of the 22*x*44*x* pollen results.

Additional analysis within cytotypes provided information that suggests plants within each ploidy group could be different cytotypes. All cytotype groups except 22*x* contain wider ranges of pollen averages than were expected. 22*x* plants can easily be distinguished from the average pollen size of other cytotypes with the average being consistent between plants. 33*x* plants contained a wider average than any of the non-mixaploid cytotypes, and contained one outlier (19_2_1). This outlier could have been due to mislabeling prior to the start of our experiment. Some 33*x* plants contained pollen averages that were more similar to those of 44*x* plants, and while this could also be due to error, it is possible that 33*x* pollen naturally contains a larger average distribution due to incomplete paring of chromosomes during meiosis resulting in gametes with higher than expected chromosome numbers.

Similar to the results of our first analysis, mixaploids contained a very large average pollen distribution between plants. 22*x*44*x* plants for example had a difference of 10 micrometers between the highest and lowest plant averages. As explained in an earlier section, 22*x*44*x* mixaploids are chimeric and are likely producing both 22*x* and 44*x* pollen. With some plants

likely producing more 22*x* pollen than 44*x* pollen and vice versa, plant averages had a large distribution. Original propagation techniques of the plant may have also been a factor in the wide range. If cuttings taken from original mixaploid plants unknowingly only selected for 22*x* tissue within the chimera, a 22*x* plant would be produced. Large differences in plant pollen averages was a phenomenon also observed with the 33*x*44*x* plants (Table 6). It is likely that the same LII pollen phenomenon seen in 22*x*44*x* plants is also occurring in these 33*x*44*x* plants. The difference however could be that the 33*x* pollen has the potential to then segregate during meiosis into 22*x* and haploid pollen. Due to the capability of these plants to produce a wide range of pollen, and having few plants of this cytotype, a larger separation of plant averages is the result. Evaluation of pollen within different cytotypes in Hydrangea also shows that there is an overlapping between cytotypes, specifically between 2*x* and 3*x* cultivars, making analyzing pollen by ploidy unreliable in certain circumstances (Jones et al., 2007).

Our experiment shows that pollen can be a good indicator of ploidy for non mixaploid plants should flow cytometry not be an available option for determining cytotypes. Additionally, with some plants having abnormally large chromosome counts, such as with *Prunus laurocerasus,* chromosome counting under a microscope could be an incredibly complex and time consuming process. Through creating a key of cytotypes for a species, plant breeders can measure pollen averages per accession to compare with a master list, therefore identifying the cytotype.

Pollen averages can also be useful for scientists studying wild-type plants. Because of wild-type diversity, pollen of plants in their native southeastern Europe can be observed to determine whether multiplication of genotypes in nature is at all possible. In the future, this information will be useful in taxonomic relations and refining intraspecific categorization.

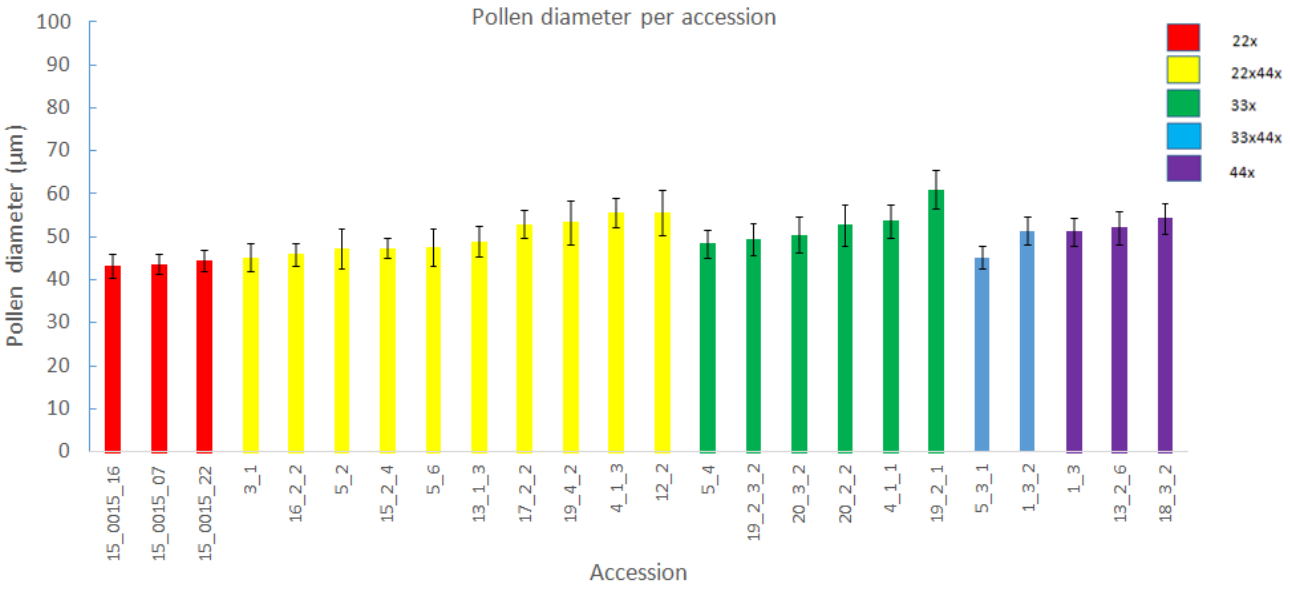

Fig. 3 Distribution of pollen diameter per accession showing that pollen averages generally increase as ploidy increases.

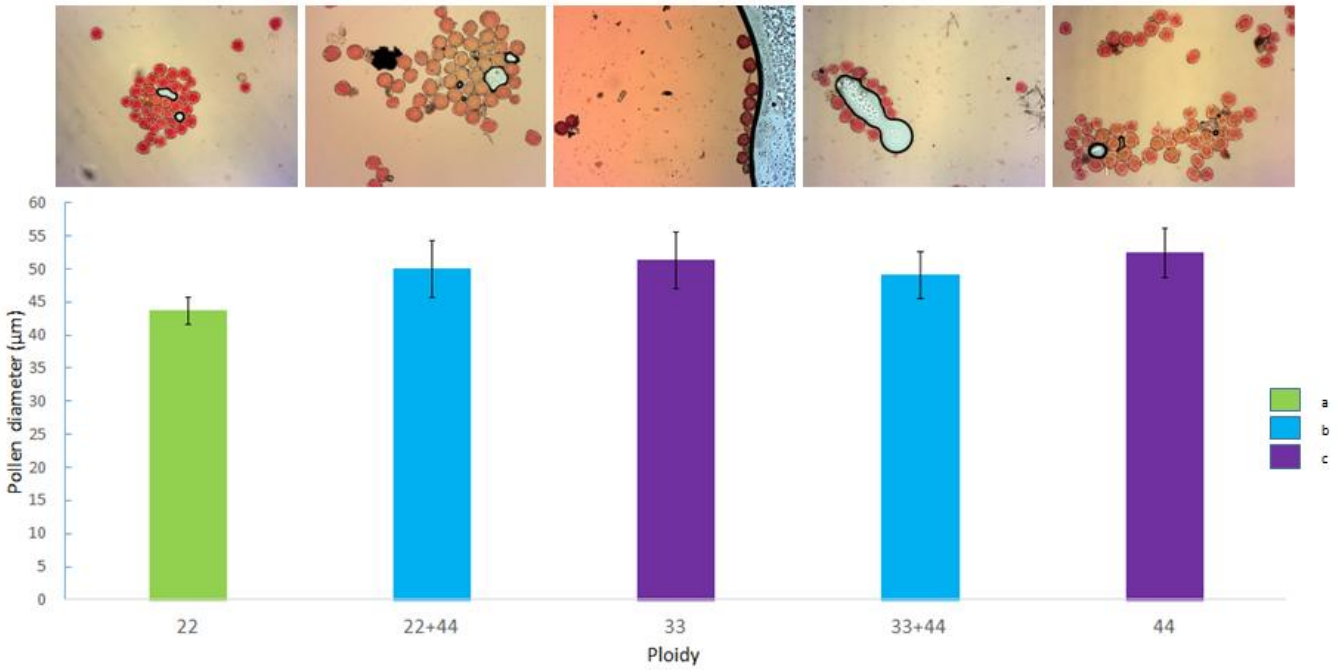

Fig. 4. Pollen averages by ploidy, showing significant groups and differences in pollen density in a given area at a 10x magnification.

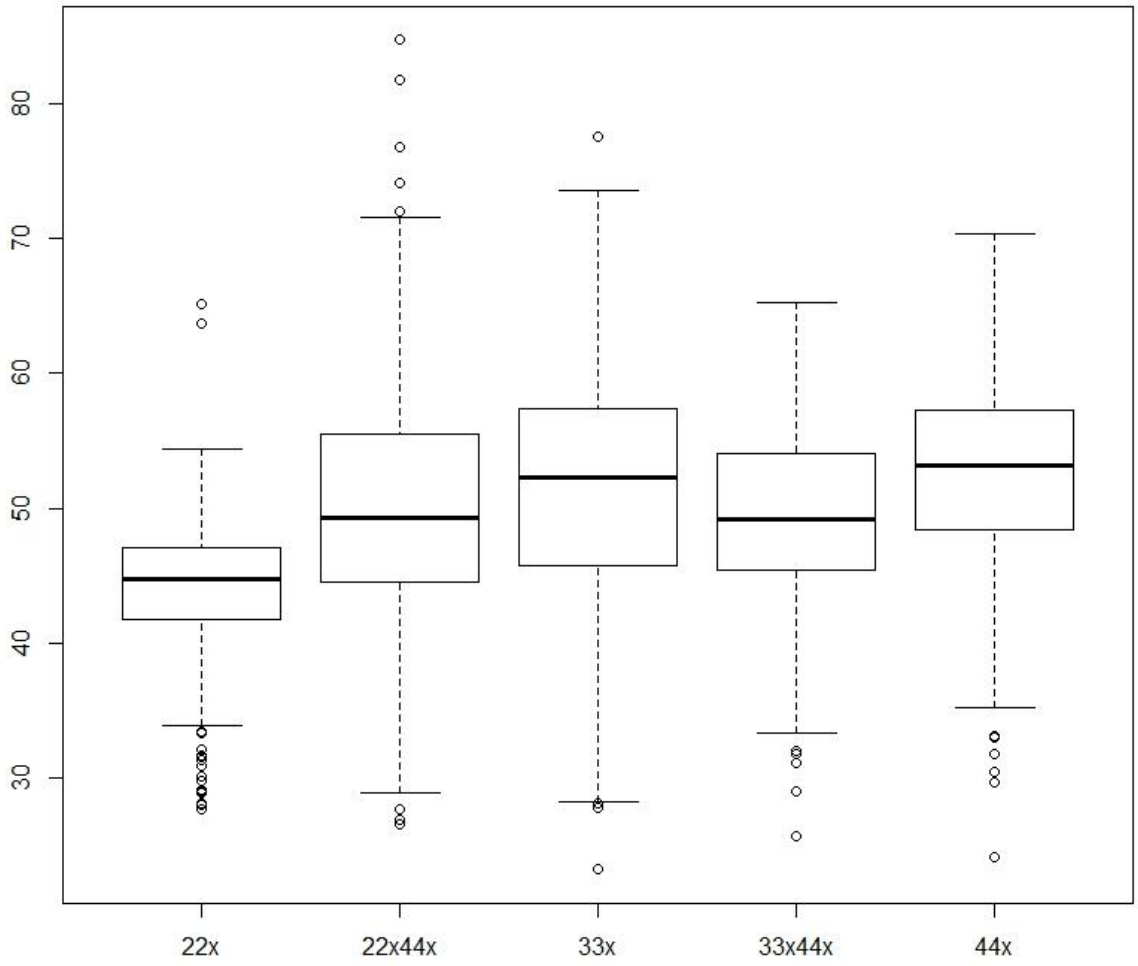

Fig. 5. Average pollen size measured in micrometers by ploidy based on individual pollen sizes. 22*x*44*x* shows largest distribution, suggesting pollen from these accessions is combinations of 22*x* and 44*x*.

Bibliography - Chapter 2

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