In Vitro Germination of Immature *Prunus lusitanica* Seed

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Abstract. **A tissue culture protocol was developed to germinate immature *Prunus lusitanica* seeds in vitro. The study was conducted by first identifying the best media for germination, followed by investigating effects of seed conditioning. In Expt. 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 6-benzylaminopurine (BA), gibberellic acid (GA$_3$), and sucrose. The longest shoots resulted from M4 (1.45 μM GA$_3$, 6 μM BA, and 30 g·L^{-1} sucrose), followed by M1 (0 μM GA$_3$, 3 μM BA, and 30 g·L^{-1} sucrose). Radicle and shoot emergence was greater than or equal to 90% for M1, M3, and M4 after a stratification treatment. In Expt. 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, nonstratified 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 before 12 WAP and will benefit from cold stratification before germinating on M1 or M4. Chemical names: 6-benzylaminopurine (BA), gibberellic acid (GA$_3$).**

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 esthetic 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*, by 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, and we have not had success after more than 10,000 attempts using hand pollination. Fruit resulting from wide, interploid crosses, as in this case [*P. laurocerasus*: 2*n* = 22*x* = 176 (Meurman, 1929), and *P. lusitanica*: 2*n* = 8*x* = 64 (Darlington and Wylie, 1956)], often abort postfertilization (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* spp. (Van Laere et al., 2007), *Prunus* spp. (Kukharchyk and Kastrickaya, 2006; Liu et al., 2007), *Rhododendron* spp. (Eeckhaut et al., 2007), and *Rosa* spp. (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 (Kukharchyk and Kastrickaya, 2006; Moore and Janick, 1983).

Prunus species exhibit intermediate physiological dormancy with a chilling requirement typically 60–90 d, and seeds that do not receive the required dormancy period have poor, if any, germination (Hartmann et al., 2011; 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, although dormancy period is even required for immature *Prunus* seeds (Kukharchyk and Kastrickaya, 2006). 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), and no fruit set has been observed in the reciprocal cross. Therefore, we 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*-inositol (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 after autoclaving.

Before addition of the agar, all media were adjusted to pH 5.8 using potassium hydroxide (KOH) and hydrochloric acid (HCl). Each 150-mm culture tube was filled with 10 mL of the medium, capped, 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 Expt. 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 appearance of red color in the fruit (≈12 WAP). In Expt. II (2015), open-pollinated seeds were collected from the same tree but collection of green fruit occurred at ≈6 and 9 WAP, whereas fruit at 12 WAP were similar to those in Expt. I and were close to appearance of red/blush color. Because flowers were open-pollinated and these trees flowered for about 2 weeks, WAP was estimated ±1 week. Seeds are fully mature at ≈16 WAP and beyond.

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, fruits were moved to a sterile laminar flow hood where the mesocarp (including the stony endocarp), and testa 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 Expt. I, after sealing tubes with parafilm M® (Bemis), seeds were placed in a cooler at 4 °C in the dark for 10 weeks before being moved to the growth area under standard growing conditions [50 μmol·m^{-2}·s^{-1} light for a 14-h light cycle (0600–2000 HR) at 25 °C]. In Expt. II, the mesocarp was removed but the testa was not, all seeds were cultured in M1 with 50 seeds per collection time. Half (25) of the seeds were placed for each collection time into cold stratification, whereas the other half of seeds were placed directly in the growth area.

Germination and morphology. The number of seeds with emerged radicles and shoots was recorded, and shoot lengths were 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 Expt. II.

Data analysis. Data were analyzed using R-studio version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). For Expt. I, shoot length was initially analyzed including nongerminated seeds, but populations lacked normality. Thus, subsequent analysis was performed only on the 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, ANOVA was performed as a three-way factorial with GA_3, 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 GA_3, BA, and sucrose as the independent variables. Model parameters for radicle and shoot emergence data analyses were tested using a likelihood ratio test. In Expt. II, radicle and shoot emergence were analyzed as in Expt. 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. 1). Radicle emergence was greater than 90% when cultured in M1–M4 (Table 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_3 had the greatest influence on shoot length at 30 g·L^{-1} than with 60 g·L^{-1} sucrose. Sucrose concentration in the media altered radicle and shoot emergence (Likelihood ratio test, $P < 0.001$). Radicle and shoot emergence were greater on media with 30 g·L^{-1} sucrose than with 60 g·L^{-1} sucrose. GA_3 also significantly increased both radicle and shoot emergence ($P = 0.03$ and $P = 0.002$, respectively), but to a lesser effect.

Medium no.1 was selected as the optimized medium for Expt. II. Although GA_3 increased radicle and shoot emergence on average, medium no.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 GA_3, 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 and 3.0 mg·L^{-1}, without using GA_3 (Sulusoglu and Cavusoglu, 2013).

In Expt. II, there was 4% radicle emergence in the nonstratified group 6 WAP but no other radicle or shoot emergence among 6 or 9 WAP treatments (Table 2). However, the 6 WAP collection time did produce callus in two seeds in the nonstratified group. Therefore, rescuing immature seed from as early as 6 weeks may be possible with further investigation into subculturing callus in this species, and may also be necessary for successful embryo rescue.

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

Table 1. Radicle emergence, shoot emergence, and shoot length of immature (12 weeks after pollination) *Prunus lusitanica* seeds without testa in Expt. 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.

Media	GA_3 (μM)	BA (μM)	Sucrose (g·L^{-1})	Radicle emergence (%)	Shoot emergence (%)	Seedlings (no.)[z]	Shoot length (mm)[y]
M1	0	3	30	100	90	27	34.7 ± 2.2 ab[x]
M2	0	6	30	93	80	24	25.5 ± 2.3 bc
M3	1.45	3	30	100	97	29	33.0 ± 2.1 bc
M4	1.45	6	30	93	90	27	42.6 ± 2.2 a
M5	0	3	60	63	47	14	25.5 ± 3.0 bc
M6	0	6	60	47	33	10	26.3 ± 3.6 bc
M7	1.45	3	60	70	60	18	27.6 ± 2.7 bc
M8	1.45	6	60	80	70	21	22.2 ± 2.4 c

GA_3 = giberellic acid; BA = 6-benzylaminopurine.

[z]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 ± least squared standard error of the mean.

[x]Mean separation based on Tukey–Kramer adjusted least squared means comparison test with $P \leq 0.05$.
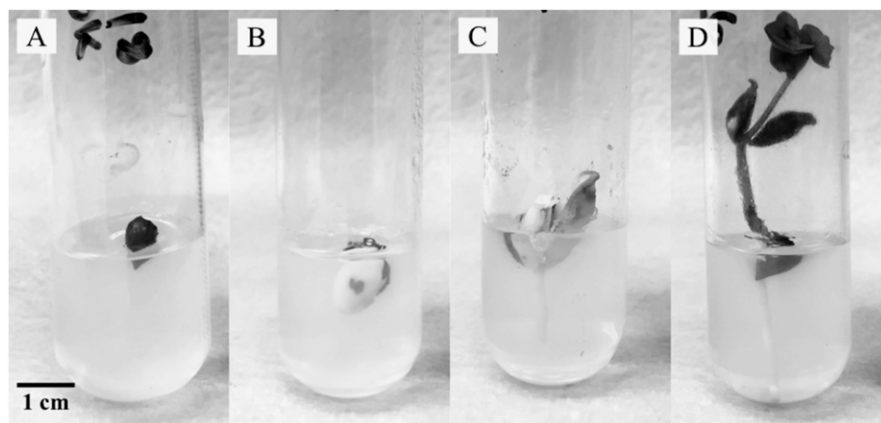
Fig. 1. Representatives of different responses observed following treatments to induce in vitro germination of cold-stratified and direct sown *Prunus lusitanica* collected 6, 9, and 12 weeks after pollination (WAP). (**A**) No development, seen in most of the 6 and 9 WAP groups, (**B**) cotyledon enlargement 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, and (**D**) radicle and shoot emergence as seen in 28% of 12 WAP cold-stratified group.

Table 2. Radicle emergence and shoots emergence of immature *Prunus lusitanica* seeds with testa cultured at three different collection times in Expt. II. Seeds were evaluated with and without 10-week cold stratification and 5 weeks under standard culture conditions.

WAP[z]	Culture method	Radicle emergence (%)	Shoot emergence (%)
6	Nonstratified[y]	4	0
6	Cold stratified	0	0
9	Nonstratified	0	0
9	Cold stratified	0	0
12	Nonstratified	36	4
12	Cold stratified	28	28

[z]WAP = weeks after pollination.
[y]Two seeds in this group produced callus.

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* and simply removing testa increased in vitro seed germination of peach by 10% (Şan and Yildirim, 2009). Arbeloa et al. (2009) reported 60% higher germination of *Prunus cerasifera* 'Myrobalan' embryos 6.5–10 mm long compared with embryos that were 0.5–2 mm, which indicates that regardless of our seed treatment the germination likely would have remained modest in the 6 and 9 WAP collections. Although shoot emergence at 12 WAP with cold stratification was higher than the groups in Expt. II, it was noticeably lower than the results of Expt. I. We speculate that it was due to the presence of a testa on seed in Expt. II.

In Expt. I, seed development allowed for easy identification and removal of the testa. In Expt. 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 Expt. I. As seeds were observed in vitro, there was phenolic accumulation on the testa which was not present in seeds during Expt. 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. Şan et al. (2014) observed dramatic increases of in vitro embryo germination without cotyledons of apricot, peach, and wild cherry; reaching 93%, 87%, and 67%, respectively.

Introgression of shothole disease resistance from *P. lusitanica* into common cherrylaurel 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 starting place for future work on embryo rescue and in vitro germination of wide cherrylaurel hybrids.

Literature Cited

Arbeloa, A., M.E. Daorden, E. García, P. Andreu, and J.A. Martín. 2009. In vitro culture of 'Myrobalan' (*Prunus cerasifera* Ehrh.) embryos. HortScience 44:1672–1674.

Bewley, J.D. 1997. Seed germination and dormancy. Plant Cell 9:1055–1066.

Darlington, C.D. and A.P. Wylie. 1956. Chromosome atlas of flowering plants. 2nd ed. Macmillian, London, United Kingdom.

De Boer, S.H. 1980. Leaf spot of cherry laurel caused by *Pseudomonas syringae*. Can. J. Plant Pathol. 2:235–238.

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

Eeckhaut, T., E. De Keyser, J. Van Huylenbroeck, J. De Riek, and E. Van Bockstaele. 2007. Application of embryo rescue after interspecific crosses in the genus *Rhododendron*. Plant Cell Tissue Organ Cult. 89:29–35.

Gudin, S. 1993. Embryo rescue in *Rosa hybrida* L. Euphytica 72:205–212.

Hartmann, H.T., D.E. Kester, F.T. Davies, Jr., and R.L. Geneve. 2011. Hartmann and Kester's plant propagation: Principles and practices. 8th ed. Prentice Hall, Upper Saddle River, NJ.

Kalinina, A. and D.C. Brown. 2007. Micropropagation of ornamental *Prunus* spp. and GF305 peach, a *Prunus* viral indicator. Plant Cell Rep. 26:927–935.

Kukharchyk, N. and M. Kastrickaya. 2006. Embryo rescue techniques in *Prunus* L. breeding. J. Fruit Ornam. Plant Res. 14:129–135.

Liu, W., X. Chen, G. Liu, Q. Liang, T. He, and J. Feng. 2007. Interspecific hybridization of *Prunus persica* with *P. armeniaca* and *P. salicina* using embryo rescue. Plant Cell Tissue Organ Cult. 88:289–299.

Meurman, O. 1929. Prunus laurocerasus L. a species showing high polyploidy. J. Genet. 21:85–94.

Moore, J.N. and J. Janick. 1983. Methods in fruit breeding. Purdue Univ. Press, West Lafayette, IN.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Pscheidt, J.W. and C.M. Ocamb (eds.). 2014. Pacific northwest plant disease management handbook. Oregon State Univ., Corvallis, OR.

Ramsey, J. and D.W. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Syst. 29:467–501.

Şan, B. and A.N. Yildirim. 2009. Seed and in vitro embryo germination in aged almond. Seed Sci. Technol. 37:365–371.

Şan, B., A.N. Yildirim, and F. Yildirim. 2014. An in vitro germination technique for some stone fruit species: The embryo isolated from cotyledons successfully germinated without cold pre-treatment of seeds. HortScience 49:294–296.

Sulusoglu, M. and A. Cavusoglu. 2013. Micropropagation of cherry laurel (*Prunus laurocerasus* L.). J. Food Agr. Environ. 11:576–579.

Trigiano, R.N. and D.J. Gray (eds.). 2011. Plant tissue culture, development, and biotechnology. CRC Press, Boca Raton, FL.

Van Laere, K., J.M. Van Huylenbroeck, and E. Van Bockstaele. 2007. Interspecific hybridisation between *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis*. Euphytica 155:271–283.

Williams-Woodward, J.L. 1998. Effect of fungicide treatment to control shot-hole disease of cherry laurel. Center for Applied Nursery Research, Dearing, GA. 10 Mar. 2015. <http://www.canr.org/98013.pdf>.