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Title: Addressing Seedling Production Challenges for Hispaniolan Pine and Snowberry

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Anthony S. Davis

The use of native plants in restoration and afforestation has increased worldwide as their benefits to habitat quality, ecosystem services and local community well-being become widely known. In many restoration and afforestation sites, the most cost-effective and efficient way to establish plants is to use seedlings. Unfortunately, there is a lack of propagation knowledge on how to germinate and grow high-quality seedlings for many native plant species, limiting their production in nurseries and their use in restoration and afforestation. The objectives for this research were to develop protocols for producing two native species important to restoration and afforestation, Hispaniolan pine (*Pinus occidentalis*) and snowberry (*Symphoricarpos albus*). Chapter 2 describes a two-part study to develop protocols to germinate and reduce seedborne fungi on *P. occidentalis*. It was found that the germination of *P. occidentalis* was unaffected by seed moisture content or cold stratification length and a 1-hour soak in hydrogen peroxide followed by a 1-hour rinse in running water was effective at reducing seedborne fungi without negatively effecting germination. Chapter 3 describes a study on the effect of nursery irrigation on the survival, morphology and physiology of *P. occidentalis* that experienced various levels of drought. Hispaniolan pine seedlings were subjected to moderate or severe drought preconditioning or were well watered (no preconditioning), then seedlings experienced no drought, moderate drought and severe drought after a simulated planting. Moderate drought preconditioning produced seedlings

with higher rates of survival on droughty sites without a decrease in seedling growth when compared to seedlings with no preconditioning or severe drought preconditioning. Chapter 4 describes a study that investigated *S. albus* seed coat permeability to water and the effect of seed moisture content on stratification requirements needed to break dormancy. *Symphoricarpos albus* has a water-permeable seed coat. The stratification treatments performed were ineffective at breaking seed dormancy and recommendations for future study are presented. These results will contribute to a growing body of knowledge on how to propagate high-quality seedlings using low-cost and nontechnical methods available to nurseries throughout the world.

Addressing Seedling Production Challenges for Hispaniolan Pine and Snowberry

by
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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.

Christina St John, Author

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CONTRIBUTION OF AUTHORS

Christina St John and Anthony S. Davis designed the studies. Christina St John collected the data and analyzed it with the assistance of Ariel Muldoon. Christina St John wrote this thesis, with critical revisions by Anthony S. Davis and Amy Ross-Davis.

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Chapter 1: General Introduction

Widespread habitat loss and the associated loss of biodiversity and ecosystem services have led to increased interest in restoration and afforestation throughout the world. As knowledge of restoration and afforestation best practices grows, recognition of the importance of using native plants rather than exotic species has increased. Native species are well-adapted to local climates and soils and can often form self-sustaining, resilient communities, reducing erosion and rebuilding topsoil while providing ecosystem services and increasing biodiversity of an area (Lamb et al., 2005; Rodrigues et al., 2009). Native forests in particular provide products for local communities, generating income opportunities in rural areas and preserving a cultural heritage that has relied on forests for generations (Byron and Arnold 1999).

In areas with landscape level deforestation with no surrounding native vegetation, the most effective restoration and afforestation technique is to plant seedlings to promote recolonization (Rodrigues et al., 2009). Unfortunately, most projects do not consider seedling specifications, such as the species required or how a seedling will respond to the environmental conditions on the site, until later in the process when the seedlings are procured (Dumroese et al., 2016; Haase and Davis 2017). There is a misconception that all seedlings are of the same quality and will survive equally well, regardless of how they were grown. This could not be further from the truth, as nursery practices have a defining role in the morphology and physiology of the seedling and its growth after planting (Duryea 1984). The incorrect pairing of seedlings to a site can result in failure. Nursery managers need specialized knowledge of plant morphology and physiology and a detailed description of the environmental conditions experienced on the site to grow seedlings with the highest likelihood for survival and growth (Dumroese et al., 2016; Haase and Davis 2017).

Despite increased interest, the use of native plants for many restoration and afforestation projects is still limited. The diversity of native species with seeds available for purchase is often low and inadequate to keep up with demand, limiting the number of species grown in nurseries (Ladouceur et al., 2018; Oldfield and Olwell 2015). The lack of established protocols on propagating many native species and the limited knowledge about site requirements and range of conditions that native plants can tolerate also prevents nurseries from propagating, and restoration and afforestation projects from utilizing, these species (Lamb et al., 2005; Davis et al., 2012; Baskin and Baskin 2005; Ladouceur et al., 2018). Without such knowledge, land managers are inclined to use exotic species that are well known and easy to procure. Unfortunately, the incorrect pairing of exotic species with a site can lead to planting failure (Cao et al., 2010). Even with high survival, sites with exotic species are less biodiverse, have altered water and nutrient cycles and provide a smaller range of ecosystem services than native forests (Rodrigues et al., 2009; Wang et al., 2012).

The lack of science-based propagation protocols for native plants is an issue for all ecosystems. In developed countries such as the United States, government agencies and universities have led efforts to determine propagation protocols and have helped to create a growing market for native plants (Bonner and Karrfalt 2008; Erickson 2008). Despite this progress, many species important to restoration and afforestation remain difficult to grow, limiting their use. Interest in propagating native plants in developing countries has increased over the past few years, but there is a lack of botanical and restoration institutions and knowledge for most noncommercial species is scarce (Baskin and Baskin 2005). Nurseries in many developing countries are small and resource-limited, where propagation techniques developed for large-scale, well-funded nurseries that rely on specialized equipment cannot be replicated (Haase and Davis 2017).

With the seeds of most native species difficult and expensive to obtain, it is imperative that nurseries know the most effective propagation methods. Many native plant seeds are dormant and will not germinate even when growing conditions are favorable. They must be exposed to certain conditions or undergo developmental changes within the seed before they germinate (Fenner and Thompson 2005; Baskin and Baskin 2014). Seed dormancy is an evolutionary trait that prevents seeds from germinating when environmental conditions are favorable for germination but likely not for growth and survival of the emergent seedling (Fenner and Thompson 2005; Baskin and Baskin 2014). This dormancy can be difficult to overcome and often leads to poor or uneven germination and waste of nursery resources. General propagation protocols for breaking seed dormancy have been developed, but the specific requirements for each species vary. It can be prohibitively expensive and time-consuming for many nurseries to experiment with finding the correct dormancy-breaking treatments for unknown species. Future research needs to refine dormancy-breaking treatments that are cost-effective and practical for many more native plants.

For restoration and afforestation projects to succeed, plants must be cultivated to be able to survive and grow in the specific environmental conditions of the site. Using the Target Plant Concept as a framework (Rose et al., 1990; Dumroese et al., 2016), nursery seedlings should have quantifiable morphological and physiological traits that can be linked to high survival and growth on the planting site. Nursery cultural practices, such as irrigation timing, affect how a seedling will respond to stresses and how quickly it can resume growth after planting (Duryea 1984). But nursery managers need information on how a specific species responds to environmental stresses and how nursery practices can modify that response. This information is lacking for many native plant species and prevents nurseries from growing seedlings that have the highest chance of survival on a restoration or afforestation site.

This research is intended to fill in gaps in propagation knowledge for two important native plants that have the potential for increased use in restoration and afforestation sites: Hispaniolan pine (*Pinus occidentalis* Swartz), an endemic pine from Haiti and the Dominican Republic, and snowberry (*Symphoricarpos albus* (L.) Blake), a shrub native to North America.

Hispaniolan Pine

The Caribbean Islands are considered a biodiversity hotspot and are a global priority for conservation due, in part, to their high level of endemic genera (Myers et al., 2000; Francisco-Ortego et al., 2007; Maunder et al., 2008). Widespread habitat loss and changes in landscape use have fragmented natural areas in the Caribbean and made conservation and restoration an immediate priority (Maunder et al., 2008).

Haiti has one of the most biologically diverse plant and animal assemblages in the Caribbean (Posner et al., 2010). Timber export, clearing land for agriculture and the production of charcoal for cooking fires has caused widespread deforestation in Haiti (Hedges 2006; McClintock 2010). While at the beginning of the twentieth century Haiti had 60% of its land covered in forests, currently only 4% of the country is forested, with no old growth forests in existence today (FAO 2010; Foxx 2012). Haiti has 1.7% of its land under federal protection for conservation, but in reality these areas have few protections and resource extraction still occurs unchecked within them (Mittermeier et al., 2005; Hedges 2006; Darrow and Zanoni 1990). Sixty-three percent of the land has a slope of greater than 20%, and deforestation has led to massive amounts of erosion and flooding, removing valuable topsoil and reducing the productivity of the land (McClintock 2010). Severe poverty and an inept and corrupt government have prevented any widespread effort to combat erosion and deforestation.

Hispaniolan pine (*Pinus occidentalis* Swartz) occurs over a wide range of habitat types and elevations in Haiti and the Dominican Republic. Forests composed of pure pine stands or mixed pine-broadleaf stands are important habitats for wildlife, making *P. occidentalis* a good candidate for use in restoration and afforestation (Darrow and Zanoni 1990; Posner et al., 2010). It grows in a variety of soil types from limestone clays to acidic gravels, thrives in shallow soils and is found at elevations ranging from 200m to 3200m (Darrow and Zanoni 1990). *Pinus occidentalis* is classified as endangered (Farjon 2013) and high elevation *P. occidentalis* forests are considered as an ecoregion of highest priority for conservation due to the high levels of endemic and endangered species found within it (Dinerstein et al., 1995). Many of the endemic birds that are found in restricted geographical locations in Haiti are found in Hispaniolan pine-mixed broadleaf forests (Posner et al., 2010). In addition to its ecological significance, *P. occidentalis* is an important cultural species. The tree has multiple medicinal uses and good wood quality (Timyan 1996; CAB International 2002). Local familiarity of the species could encourage its use in restoration and afforestation projects (Davis et al., 2012).

Although there are some nurseries in Haiti and the Dominican Republic growing *P. occidentalis*, very little information is known about the ecology, genetic variation and propagation of this species (Darrow and Zanoni 1990). Many nurseries focus on seedling quantity rather than quality, resulting in seedlings that do not survive after planting (Hubbel et al., 2018; Haase and Davis 2017). There are currently no science-based propagation protocols for germinating *P. occidentalis*. The isolated location of the remaining *P. occidentalis* stands makes obtaining seeds difficult. It is important to develop the most effective and efficient seed pretreatment protocols for propagating *P. occidentalis* when nurseries obtain seed. The research in this study will determine the seed moisture content that leads to the highest germination rate, the influence of cold stratification length on germination rate and the efficacy of readily applicable treatments to control seedborne fungi.

In addition to the lack of germination protocols, the drought tolerance of *P. occidentalis* is also unknown. The large historical range occupied by *P. occidentalis* requires that nurseries grow seedlings that can survive under many different precipitation regimes. In addition, climate projections for later this century predict that Haiti and the Dominican Republic will see a decrease in precipitation, especially during what is now considered the early wet season (Karmalkar et al., 2013). Nurseries producing *P. occidentalis* seedlings for restoration and afforestation will need to be able to grow seedlings that can survive prolonged drought. The second goal of this research is to determine how the development of *P. occidentalis* morphology is affected by watering schedule and whether the watering schedule a seedling experiences during its first season of growth in the nursery influences its survivorship and growth after planting.

Snowberry

Snowberry (*Symphoricarpos albus*) is a small to medium sized shrub found throughout most of North America (Bell and Dempster 2012; USDA NRCS 2017). *Symphoricarpos albus* provides food and shelter for wildlife and is used in the restoration of disturbed sites, especially along riparian areas (McWilliams 2000). Despite having germination pretreatment protocols in multiple propagation manuals, germination rates of *S. albus* from seed can be low, and most nurseries propagate the species from cuttings. Cuttings are a form of asexual plant propagation, which uses segments of a parent plant to create clones. Although this is a cost-effective method for producing plant material, a crop produced from cuttings has lower genetic diversity than that produced from seeds. High genetic variability within plant populations is correlated with restoration success (Kettenring et al., 2014; Hughes et al., 2008). More research is needed on effective methods for breaking dormancy in *S. albus* to achieve adequate germination.

Other species of snowberry have morphophysiological dormancy, where the embryo is underdeveloped at dispersal and compounds or a structure within the seed prevents germination (Fenner and Thompson 2005; Hidayati et al., 2001). There is debate about whether this genus has physical dormancy, or the seed coat is impermeable to water (Walker 2008; Hidayati et al., 2001). *Symphoricarpos albus* requires up to nine months of stratification to break seed dormancy, including both warm moist and cold moist stratification (Walker 2008; Dirr 1998; Rose et al., 1996). The purpose of this research is to determine if *S. albus* has physical dormancy (a water impermeable seed coat) and if the stratification requirements of *S. albus* are affected by the moisture content of the seed when placed in stratification.

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Chapter 2: Developing a germination protocol for Hispaniolan pine (*Pinus occidentalis* Swartz)

Abstract

Hispaniolan pine (*Pinus occidentalis* Swartz), an endemic species from Haiti and the Dominican Republic, has the potential for widespread use in restoration and afforestation, but the lack of reputable propagation knowledge limits the use of this species. Recognizing that seedlings will be produced in resource-limited environments, a suite of studies were completed to inform how to effectively germinate seeds in a manner that will likely lead to successful nursery culture. Specifically, this work sought to address that there is no formal guidance regarding a) seed moisture content at sowing, b) use of stratification to overcome dormancy, and c) the efficacy of treatments to control seedborne fungi. Germination in *P. occidentalis* is not affected by seed moisture content or cold stratification length. This important finding warrants a shift from the current practice of soaking seeds in standing water. No chemical treatments negatively affected the germination rate and a 1-hour soak in 3% hydrogen peroxide solution followed by a 1-hour running water rinse significantly reduced the presence of fungi on germinated seeds. Together, these studies reduce the risk of seed loss to fungi (both seedborne and waterborne) and provide for simple and replicable practices in nurseries in Haiti and the Dominican Republic.

Introduction

Seed Dormancy in Western Hemisphere Pines

Pines (*Pinus* spp.) have a diverse array of dormancy types, with most temperate and tropical American pines having either physiological dormancy or are non-dormant (Baskin and Baskin 2014). Physiological dormancy occurs when the embryo is fully developed and can uptake water, but chemicals within the seed or a physical structure in the seed prevents germination (Fenner and Thompson 2005). Physiological dormancy in pines is mainly coat-induced dormancy (Cooke et al., 2002; Kolotelo et al., 2001). The embryo is fully mature but the seed coat and nucellar cap, which covers the micropyle end of the seed where the radicle emerges, provides mechanical restraint against radicle protrusion (Cooke et al., 2002; Downie et al., 1998; Carpita et al., 1983). Seeds can germinate when the embryo can create enough force to push through the seed coat, nucellar cap and, to a lesser extent, the megagametophyte, the maternal storage tissue that surrounds the embryo (Barnett 1976).

The pine seed coat restricts the amount that the megagametophyte and embryo can swell, limiting the seeds ability to absorb sufficient water for germination (Cooke et al., 2002; Tillman-Sutela and Kauppi 1995; Barnett 1976). In pines found in the southern United States, the constraint imposed by the seed coat is related to the weight of the seed coat relative to total seed weight and that ratio is directly related to the degree of dormancy seen in seeds. Longleaf pine (*P. palustris*), which does not require treatments to germinate and is considered the least dormant southern pine, has the smallest proportion of seed weight composed of the seed coat and can uptake water rapidly with the seed coat intact. Loblolly pine (*P. taeda*), which is considered the most dormant southern pine and requires the longest treatment to break dormancy, has the highest proportion of seed weight composed of the seed coat and can absorb only a limited amount of water while the seed coat is intact (Barnett 1976).

The permeability of seed tissues to water also plays a role in dormancy and germination. Imbibition is the process by which seeds uptake water necessary to initiate cellular activity, break dormancy and germinate (Landis et al., 1998). The seed coat is permeable to water, but the nucellar cap and a papery layer between the seed coat and nucellar cap are impermeable to water (Cooke et al., 2002; Tillman-Sutela and Kauppi 1995; Barnett 1976). These tissues partially surround the megagametophyte and embryo, forcing water to diffuse through the center of the megagametophyte and embryo to the radicle, which is the last section of the seed to imbibe (Cooke et al., 2002; Tillman-Sutela and Kauppi 1995). By slowing the rate of water uptake by the radicle, the rest of the seed is imbibed and metabolizing compounds necessary for germination before the radicle has imbibed and is ready to push through the seed coat (Tillman-Sutela and Kauppi 1995).

Seed Treatments to Overcome Dormancy

Many conifer seeds are considered “shallowly dormant” as they require stratification under cold moist conditions to germinate (Jones and Gosling 1994). The two most common seed pretreatments used to break dormancy and allow germination are soaking seeds in water to reach a specific moisture content and placing seeds in cold stratification (Krugman and Jenkinson 2008; Kolotelo et al., 2001). Cold stratification is the process of placing imbibed seeds into cold temperatures, usually in a refrigerator, for a certain amount of time (Kolotelo et al., 2001).

Water performs a critical role within seeds. It is required for the proper functioning of enzymes, acts as a solvent within which chemical reactions can occur and molecules can be transported and is a reactant in the breakdown of storage compounds (Woodstock 1988). There is also evidence that some pine species contain germination inhibitors in the seed coat that are removed during soaking (Martinez-Honduvilla and Santos-Ruiz 1978). A seed’s ability to break dormancy and its response to changes in

temperature is dependent on moisture level (Vertucci and Leopold 1986). The rate of imbibition varies across conifer species (Dumroese 2000; Kolotelo et al., 2001). Conifer seeds that are sown dry can have delayed germination because of the time it takes for them to absorb enough water from the potting media to initiate germination (Kolotelo et al., 2001).

Seed moisture levels are important for the effectiveness of cold stratification. The ideal moisture content during stratification varies by species (Kolotelo et al., 2001). Species such as Sitka spruce (*Picea stichensis*), western hemlock (*Tsuga heterophylla*) and ponderosa pine (*P. ponderosa*) require low moisture contents (<30%) during stratification. Lodgepole pine (*P. contorta*) seed requires a moisture content between 30 and 32% for cold stratification while Douglas-fir (*Pseudotsuga menziesii*), western white pine (*P. monticola*) and grand fir (*Abies grandis*) require moisture contents greater than 32% (Kolotelo et al., 2001). Some tropical pines, such as *P. maximinoi*, *P. montezumae* and *P. leiophylla*, have increased germination rates if soaked before sowing, while the germination rate for tropical pines such *P. caribaea* and *P. patula* as are not affected by soaking (Vozzo 2002; Donald 1981; Ghosh et al., 1974).

Many conifer seeds require cold stratification at 1-5°C (Gosling 1998). Stratification requirements vary among species and even among seed lots of the same species (Landis et al., 1998; Carpita et al., 1983). Cold stratification increases the growth potential of the embryo and allows it to overcome the mechanical restraint of the seed coat (Carpita et al., 1983). In loblolly pine (*P. taeda*), the rate of radicle expansion was faster for seeds that had undergone cold stratification (Carpita et al., 1983). The exact mechanism for this physiological change is unclear. Stratification does not change the seed coat structure or affect the breakdown of storage proteins in the megagametophyte but does slightly alter the composition of proteins that are being created in the embryo (Cooke et al., 2002). It has been suggested that cold stratification in western white pine (*P. monticola*) may allow for the

production and accumulation of oxygen-rich compounds needed for germination, such as ATP, without the expenditure of storage compounds that are also necessary for germination (Dumroese 2000).

Conifer seeds can often germinate faster, more evenly and over a wider range of temperatures after they have been placed in cold moist stratification (Kolotelo et al., 2001; Landis et al., 1998; Jones and Gosling 1994). This allows nurseries to sow seeds earlier in the growing season and produce higher quality seedlings with similar morphology (Barnett and Pesacreta 1993; Moreno 1985). *Pinus taeda* seedlings that germinated from stratified seeds were, on average, 0.1 foot taller than seedlings that germinated from unstratified seeds due to an increase in the speed of germination (Barnett and McLemore 1984). In addition, *P. taeda* seedling diameter was decreased between 1.2 and 1.7% for every day that germination was delayed (Boyer et al., 1985). Stratification does not have the same effect on morphology for all conifers. For slash pine (*P. elliottii*), stratification increased germination speed by two days but did not affect seedling morphology (Barnett and McLemore 1984). Stratification is detrimental to the germination of longleaf pine (*P. palustris*), Caribbean pine (*P. caribaea*) and Mexican yellow pine (*P. oocarpa*) (Barnett and Jones 1993; Moreno 1985)

Seedborne Pathogens and Their Control

All seeds carry a microbiota which contains both beneficial and harmful microorganisms (Agarwal and Sinclair 1997). A seedborne pathogen is defined as an organism that is carried on or within seeds and has the potential to cause disease in the seed or emerging seedling (Maude 1996; Cram and Fraedrich 2009). It has been estimated that up to 10,000,000 viable spores can be found on one gram of conifer seed (Rees and Phillips 1994). Seeds can be infected while developing on the parent plant or after maturity and dispersal. Pathogens also spread among seeds during cleaning, storage, imbibition and cold stratification (Kolotelo et al., 2001).

Some seedborne pathogens will infect the seed during storage, killing damaged and low-vigor seeds and reducing overall germination (Cram and Fraedrich 2009). Seedborne pathogens can spread from the seed coat to the seedling during germination and growth. Pathogens can kill seedlings before the seedling emerges aboveground (pre-emergence damping-off) or after it emerges (post-emergence damping-off, shoot blight or root rot) (Cram 2017; Cram and Fraedrich 2009). Other pathogens will only cause temporary growth reduction and shoot deformation, but healthy seedlings are able to outgrow the pathogens (Rees and Phillips 1994). Some common conifer seedborne fungal pathogens that cause seed or seedling death are in the genera *Fusarium*, *Diplodia, Alternaria* and *Rhizoctonia* (Talgo et al., 2010; Lilja et al., 1995; Rees and Philips 1994).

Some pathogenic seedborne fungi establish on conifer seeds only during storage. *Aspergillus* and *Penicillium* are the most common storage fungi (Agarwal and Sinclair 1997). Storage fungi are typically saprophytes contaminating the surface of seeds but will infect internal tissues if there is a wound opening. They cause seed deterioration during storage and do not affect the seed or seedling once planted. Storage fungi produce compounds that can accelerate the process of seed aging, which leads to decreased vigor and may eventually lead to seed death (Agarwal and Sinclair 1997).

Preventing seeds from becoming contaminated and controlling pathogen levels on seeds once they become infected requires a combination of cultural sanitation practices and chemical treatments. No single method has been discovered that can control all pathogens and the diseases they cause. Seed producers and nursery staff must take an integrated management approach and look at different steps that can be taken at all stages of seed collection, storage and sowing. Seed handling during cleaning, storage and seed pretreatments is often when most pathogens increase on seeds and spread among seed lots (Littke 1996).

One of the most efficient and effective ways to control seedborne pathogen levels is by disinfecting seeds (Kolotelo et al., 2001). Disinfecting treatments are effective at removing contaminating pathogens on the outside of seed coats but are not effective at removing infections that have penetrated the internal tissues of the seed (Wenny and Dumroese 1987). Two chemical treatments commonly used to disinfect conifer seeds are soaking in hydrogen peroxide or bleach (sodium hypochlorite). Hydrogen peroxide is good at reducing pathogen levels and is less phytotoxic than other chemicals used (James and Genz 1981). It is the most common chemical used to disinfect conifer seeds in North America (Kolotelo et al., 2001). Hydrogen peroxide also stimulates germination in some conifer species, such as ponderosa pine (*P. ponderosa*) and Douglas-fir (*Pseudotsuga menziesii*) (Dumroese et al., 1988; James and Genz 1981). For some thin-coated or porous-seeded species, performing the hydrogen peroxide soak after imbibition and stratification, when the seeds are full of water, prevents hydrogen peroxide from entering the seeds and damaging internal tissues (Dumroese et al., 1988). Bleach is another commonly used disinfectant chemical, though it is more likely to damage seeds than hydrogen peroxide. Depending on the length of the soak, bleach can cause seed etching and can negatively affect the viability of thin-coated conifers such as true firs, larches and spruce (Wenny and Dumroese 1987). Unlike hydrogen peroxide, bleach does not penetrate undamaged seed coats and the treatment can be applied either before or after imbibition and stratification (Dumroese et al., 1988). Recommendations for the composition of bleach solutions range from 3:2 water to bleach to 5:1 water to bleach, depending on the species (Kolotelo et al., 2001; Dumroese et al., 1988).

The easiest treatment is a running water rinse and can be used while seeds are being imbibed before placed in stratification. A running water rinse is more effective at removing spores than a still water soak while allowing seeds to fully imbibe (James and Genz 1981). A running water rinse does not negatively affect the viability of seeds and is good for species that have thin and permeable seed coats

that are sensitive to chemical treatments (Dumroese et al., 1988). One pitfall to a running water rinse is that this treatment can spread spores if uninfected seeds are soaked with infected seeds (Kolotelo et al., 2001). A running water rinse is not effective at removing all spores and can still lead to disease in treated seeds and seedlings (James 1987).

Cold stratification has varying effects on the incidence of seed and seedling death due to seedborne pathogens. Mittal et al. (1987) found that cold stratification of any duration increased fungal infection on white spruce (*Picea glauca*) and eastern white pine (*P. strobus*) seedlings, which affected seedling quality. On heavily infected seed lots, cold stratification can decrease seedling death due to an increase in seedling vigor and rate of growth. Mittal and Wang (1993) found that when *P. strobus* seeds were inoculated with fungi, seedlings grown from seeds that did not undergo cold stratification were more likely to be killed by fungi than seedlings from seeds that underwent cold stratification. Controlling moisture levels on the seed coat during stratification is the most effective way of preventing fungal growth (Kolotelo et al., 2001; Downie et al., 1998)

The presence of fungi on seeds is not always an indicator that the emerging seedling will be infected. Nursery practices can affect whether fungi can invade or kill seedlings (Cram 2017; Landis 1989). The type of potting media can affect whether pathogens on the seed remain alive after sowing and infect seedlings. Sphagnum peat moss is the most commonly used growing media in North American nurseries (Landis 1990) and has been shown to contain beneficial microorganisms that can reduce damping off from pathogens such as *Pythium*, *Rhizoctonia* and *Alternaria* (Wolffhechel 1988; Tahvonen 1982), although levels of beneficial microorganisms varied among peat sources (Tahvonen 1982). Peat was not effective at suppressing *Fusarium* (Tahvonen 1982). High humidity and potting media moisture and increased nitrogen levels can encourage the growth of pathogens and increase the

risk of damping off, so allowing media to dry out and fertilizing when seedlings are older and less vulnerable to seedborne pathogens can decrease the incidence of disease (Cram 2017).

Pinus occidentalis is a critical species for restoration and afforestation in Haiti and the Dominican Republic, but the isolated location of remaining stands makes seed procurement difficult (Hubbel et al., 2018; Posner et al., 2010). Once seed is obtained, it is imperative that nurseries know the most effective methods for propagation to grow high-quality seedlings. Protocols must be science-based and utilize materials that are available and affordable to local nurseries (Haase and Davis 2017). There has been no study to determine how treating seed prior to sowing influences *P. occidentalis* germination. The objectives of this research were to test the effects of seed moisture content and cold stratification length on the germination rate of *P. occidentalis* and to investigate the effect of different sanitation treatments on germination rate and presence of fungi on germinated *P. occidentalis* seeds. It was hypothesized that seeds soaked to 35% moisture content and that received no cold stratification would have the highest germination rate and that soaking seeds in hydrogen peroxide prior to sowing would decrease the number of germinated seeds with fungi while maintaining an adequate germination rate when compared to no treatment.

Methods

Pinus occidentalis seeds were obtained from the Boca de Nigua National Seed Bank, Dominican Republic (collected from a single population near Jarabacoa in La Vega Province, Dominican Republic in September 2013). The seed lot was stored at 5°C with desiccant until use. Seeds used were randomly selected from the seed lot using seed industry protocol for hand sampling (AASCO 2006). In July 2017, a tetrazolium test performed at the Oregon State University Seed Lab (Corvallis, OR, USA) found the seed lot to have 84% viability. Three studies were completed and described below.

Imbibition Study

The rate of water uptake was found for *P. occidentalis* seeds to determine soaking lengths needed to obtain the desired seed moisture contents for the following study. The water content for seeds in dry storage was determined by drying three samples of 25 seeds each in an oven at 103°C for 26 hours (Elias et al., 2012). To create the water uptake curve, seven samples of 25 seeds each were randomly selected and the starting weight of each recorded. Each sample was placed in a mesh bag and submerged in aerated distilled water. Each sample was blotted dry and weighed every 3 hours for 12 hours, then every 12 hours until hour 84, then again at 108 and 130 hours.

Moisture Content and Cold Stratification Study

The seed moisture contents and lengths of cold stratification tested were selected based on previous research that determined the optimum moisture content for the germination of temperate conifer seeds and the optimum length of cold stratification of some tropical pines (Vozzo 2002; Kolotelo et al., 2001). Five levels of seed moisture levels were tested: (1) 5% (dry seed), (2) 27%, (3) 31%, (4) 35% (control) and (5) 39%. Five levels of cold stratification length were tested: (1) 0 days (control), (2) 7 days,

(3) 14 days, (4) 21 days and (5) 28 days. The 35% moisture content, 0 days cold stratification is the current seed pretreatment protocol used in nurseries and was considered the control treatment (pers. comm. W. Placido Made). All combinations of five seed moisture levels and five lengths of cold stratification were compared, resulting in a total of 25 treatment combinations.

Seeds were randomly selected and divided into 75 replicates containing 25 seeds each. Every replicate was randomly assigned a seed moisture level and cold stratification length combination, with three replicates assigned to each treatment combination. Each replicate of 25 seeds was considered an experimental unit and placed into a mesh bag. Seeds were cleaned by soaking in a 3:1 hydrogen peroxide (3% concentration): water solution for 5 minutes then rinsed in running water for 10 minutes. The bags were placed in distilled water, aerated with a small fish tank pump, for differing amounts of time to get the desired moisture content. Soaking times were determined using the water uptake curve best fitted line equation from the previous study. Once removed from soaking, each bag was blotted dry and underwent cold stratification using the standard naked stratification protocol for temperate conifers, where bags were hung in a single refrigerator (average temperature of 6°C) with a dish of water below the seeds (Kolotelo et al., 2001).

Once the seeds had completed their assigned stratification period, the seeds from a single bag were removed and placed into a 9 cm plastic petri dish on top of 2 layers of Whatman No. 1 filter paper moistened with 2.5 mL of distilled water. This was repeated for each bag of seeds. Dishes were placed into a single germination chamber (Hoffman Manufacturing, Corvallis, OR, USA) set at 25°C/15°C at 12 hour intervals with 12 hours of light. Germination and dish moisture levels were monitored daily for 30 days. Distilled water was added to a petri dish if that dish fell below 75% moisture content, determined by weight, to maintain uniform moisture status in every dish. Seeds were considered germinated when the radicle exceeded 1 mm in length and pointed downward. Seeds were removed from the dish once

germinated and the number of germinated seeds were recorded daily. Seeds with fungi were wiped clean and placed back into the dish. The location of the petri dishes within the germination chamber was randomized daily.

Sanitation Study

The seed sanitation treatments used were based on a literature search of the most effective methods to control pathogens on temperate conifer seeds while taking into consideration materials that could be obtained in Haiti. The list of treatments can be found in Table 2.1. The treatments used varying concentrations of bleach and hydrogen peroxide and varying rinse times in running water, as well as a running water only rinse and a control (no treatment). Seeds were randomly selected and divided into 44 replicates of 25 seeds each. Each replicate of 25 seeds was considered an experimental unit and each sanitation treatment contained 4 replicates. Once the replicates of seeds had undergone their assigned sanitation treatment, the same germination protocols from the previous study were used. In addition to recording germination daily, the number of germinated seeds with fungi was recorded. After the end of the germination experiment, all ungerminated seeds were sent to the Oregon State University Plant Clinic (Corvallis, OR, USA) and all fungi with reproductive structures were identified to genus.

Statistical Analysis

All analyses were done with R (Version 3.4.3, The R Foundation for Statistical Computing, 2017). For every experiment, all dishes are assumed to be independent. All effects of interest were estimated from the corresponding models. Differences among treatments for each parameter were analyzed using either one-way or two-way ANOVAs and comparisons were made between the control and treatments using Dunnett's test ($\alpha = 0.05$).

The moisture levels needed for the moisture content and cold stratification length study occurred between 3 and 24 hours of imbibition, so only data between those times was used to obtain the best fitted line for the rate of water uptake. A time series linear mixed model was used to determine the seed moisture levels during imbibition, and a best fitted line equation was created using this model.

A generalized linear model using a quasi-binomial distribution with the logit link was used to test the effect of seed moisture content and cold stratification length on the odds of germination. The mean germination time (MGT) was determined for each moisture level and stratification length combination using the formula from Bewley and Black (1985):

$$MGT = \Sigma(T*N)/\Sigma(N)$$

T = Time (days)

N = Number of seeds germinating on day T

A linear model was used to test for differences in the mean MGT between moisture content and stratification length treatments.

A generalized linear model using a binomial distribution with the logit link was used to test the effect of the seed sanitation treatment on the odds of germination. A generalized linear model using a quasi-binomial distribution with the logit link was used to test the effect of the seed sanitation treatment on the odds of a germinated seed having fungi. A linear model was used to test for differences in MGT among sanitation treatments and the control.

Results

Imbibition Study

Water uptake (Figure 2.1) took 5 hours and 49 minutes to achieve the 27% seed moisture content, 8 hours and 34 minutes to achieve 31% seed moisture content, 12 hours and 2 minutes to achieve 35% seed moisture content and 17 hours and 25 minutes to achieve 39% seed moisture content. The equation to determine seed moisture content at a given time between 3 and 24 hours is:

Moisture content (% fresh weight) = $16.302436 + 2.113130*time - 0.046526*time^2$

Moisture Content and Cold Stratification Study

There is no evidence that the odds of a seed germinating are statistically different among seed moisture content levels ($p = 0.96$) or cold stratification lengths ($p = 0.27$), nor was there evidence of an interaction between moisture content and cold stratification length ($p = 0.94$) (Table 2.2). The ratios of estimated odds of germination, as well as 95% confidence intervals, for specific seed moisture contents (Figure 2.2) and lengths of cold stratification (Figure 2.3) both showed little treatment effect.

Mean germination time did not differ among moisture content levels ($p = 0.23$) but did differ statistically among stratification lengths ($p < 0.0001$). There was no interaction between moisture content and cold stratification length ($p = 0.62$) (Table 2.2). The MGT for 14, 21 and 28 days of cold stratification were significantly longer than for 0 days cold stratification ($p = 0.039$, $p < 0.0001$ and $p = 0.047$, respectively). The odds of germination and MGT for all moisture content and stratification lengths tested are found in Table 2.3.

Sanitation Study

There is no evidence of a statistically significant difference in the odds of a seed germinating among any sanitation treatment and the control ($p = 0.16$) (Table 2.4 and Figure 2.4). There is statistical evidence of a difference in MGT among different sanitation treatments ($p = 0.0015$). Treatment 5, the 10 minute soak in 2:3 bleach:water solution followed by a 30 minute running water rinse, was the only treatment to be statistically different and had a larger MGT than the control treatment ($p = 0.0013$) (Table 2.4).

There is evidence of a difference in the odds of a germinated seed having fungi when subjected to different sanitation treatments ($p < 0.0001$) (Figure 2.5). Treatment 7, the 1-hour hydrogen peroxide soak followed by a 1-hour running water rinse, was the only treatment to be statistically different from the control treatment and had a decrease in germinated seeds with fungi ($p = 0.044$) (Table 2.4). For seeds that did not germinate, 15 genera of fungi were identified (Table 2.5).

Discussion

Germination and seedling production protocols are a critical early step needed for producing high quality native plants for restoration and afforestation projects (Haase and Davis 2017). Dumroese et al. (2016) highlight the need for nursery production methods, including the source of plant material, to be considered in designing a successful restoration or afforestation project. Hubbel et al. (2018) discuss the role of growing media and container selection in *P. occidentalis* seedling development but did not identify key considerations in seed handling to maximize the likelihood of producing high quality seedlings for restoration and afforestation.

The germination tests performed suggest that *P. occidentalis* has a nondormant seed. Imbibing seeds prior to sowing and cold stratification did not affect germination. Germination rates seen during the experiment were similar to the 84% viability of the seed lot determined just prior to the study. While future research is needed to illuminate dormancy patterns in tropical pines, it is possible the lack of dormancy in *P. occidentalis* could be related to the weight of the seed coat relative to the rest of the seed, though this was not researched in the current study. Pines with more dormant seeds have larger proportions of the total seed weight composed of the seed coat, which reduces the rate of water uptake (Barnett 1976). Highly dormant temperate pines, such as western white pine and sugar pine, require at least 72 hours of soaking to reach full imbibition (Dumroese 2000; Baron 1978). *Pinus occidentalis* seeds reached full imbibition in 20.5 hours, which is similar to longleaf pine (*P. palustris*), a species that also germinates without cold stratification or imbibition and has the smallest seed coat weight ratio of southern pines (Barnett 1976). It is possible that *P. occidentalis* has a similar seed coat structure to *P. palustris* that allows for faster imbibition and requires less force from the embryo to penetrate.

Different moisture content levels in sown *P. occidentalis* seeds did not lead to a statistically significant difference in germination rate or mean germination time (MGT). In addition to testing for a statistically significant difference, this study wanted to determine if there was a biologically significant difference in germination rate or MGT that could affect nursery production. For this study, a biologically significant response was based on the International Seed Testing Association's tolerance tables for allowable differences between the results of two germination tests (ISTA 2018). For two tests with 100 seeds, a significant difference would be a change in mean germination rate of 14 percentage points or greater, which would be indicated by an odds ratio below 0.53 or above 2.58. The confidence intervals for all odds ratios comparing each moisture content level to the control indicate that there is no biologically significant difference in germination rate among seed moisture levels (Figure 2.2). The biologically significant response in MGT would be a change of 5 days from the control (Mexal and Fisher 1987), which no treatment showed (Table 2.3).

Unlike this study, most research on conifer seed treatments have found an increase in germination rate and speed when seeds are imbibed prior to sowing (Himanen et al., 2013; Kolotelo et al., 2001; Barnett 1976), though *P. palustris* shows a decrease in germination rate when soaked for progressively longer periods of time (Barnett and Jones 1993). Sowing seeds dry could have a positive production benefit for nurseries located in Haiti and the Dominican Republic, many of which do not have access to clean running water or dedicated workforce with advanced training in seed handling (Haase and Davis 2017; pers. comm. A. Davis). Eliminating the time seeds spend soaking in contaminated still water could reduce the level of pathogens found on the seeds and reduce resource use.

Although there was no statistical difference in germination rates among cold stratification lengths tested, for the 7 days and 14 days cold stratification lengths, there is a potential that those treatments could decrease the odds of a seed germinating in *P. occidentalis* (Figure 2.3). There was no

biologically significant difference in MGT even though there was a statistically significant increase in MGT in 14 day, 21 day and 28 day stratification treatments (Table 2.3). Common nursery protocol is to perform the longest stratification period that does not decrease germination rate, since most species are able to germinate more uniformly and over a larger range of temperatures after stratification (Barnett 2008; Downie et al., 1998; Moreno 1985). Although this may be practical in North American nurseries that have access to reliable equipment and electricity, this would be a difficult practice to implement at some nurseries in Haiti and Dominican Republic, where electricity may be intermittent or unavailable and refrigeration equipment prohibitively expensive. In addition, the benefits of being able to germinate over a wide range of temperatures may not be as relevant in Hispaniola, where seasonal temperatures do not fluctuate as widely as in temperate North America. Without a clear benefit to germination rate or MGT, the process of stratifying seeds may not be practical for *P. occidentalis*.

Among the sanitation treatments, although there was no statistical difference in germination rates, there was a potential biological difference (Figure 2.4). There is no pattern to what treatment could potentially increase or decrease germination, and the potential biological difference could be due to large germination rate confidence intervals due to the small samples used (4 replicates per treatment). MGT increased only for treatment 5, the 10 minute soak in 2:3 bleach: water mix followed by a 30 minute running water rinse (Table 2.4). The confidence interval for the MGT for treatment 5 contained values larger than 5 days when compared to the control, which is the biologically significant value that could lead to seedlings being smaller than seedlings whose seeds had received other treatments (Mexal and Fisher 1987).

The only treatment to have a statistically significant effect on the number of germinated seeds with fungi was treatment 7, the 1-hour hydrogen peroxide (3% solution) soak followed by a 1-hour running water rinse (Figure 2.5). Past studies have found bleach and hydrogen peroxide to be effective

at decreasing the level of fungi on seeds (James and Genz 1981; Wenny and Dumroese 1987; Dumroese et al., 1988), but only 1 of the 9 treatments in this study that used those chemicals had a statistically significant difference in germinated seeds with fungi. Although there was no difference in fungi, the bleach and hydrogen peroxide solutions did eliminate fungi that are known to cause seed or seedling death in otherwise healthy seedlings (*Alternaria, Fusarium* and *Rhizoctonia*) (Table 2.5) (Talgo et al., 2010; Lilja et al., 1995; Mittal and Wang 1993). Most of the treatments contained fungal genera that are weakly pathogenic and can cause damping off in seeds or seedlings (*Botryosphaeria, Chaetomium*, *Epicoccum* and *Ulocladium*) (Talgo et al., 2010; Lilja et al., 1995; Rees and Phillips 1994). These genera can cause disease when seeds are damaged during cleaning or storage or may reduce seedling vigor during early growth, but do not affect healthy seeds or seedlings (Cram and Fraedrich 2009; Kolotelo et al., 2001). Species in the genera *Aspergillus, Cladosporium*, and *Penicillium* are considered storage fungi. They are saprophytic, feeding on decaying or dead seed tissue, and are associated with poor quality seeds or seeds that have been stored incorrectly (Agarwal and Sinclair 1987; Rees 1983). It is not surprising that this seed lot would contain storage fungi as it has been transferred between locations multiple times and has experienced changes in storage temperature and humidity, allowing fungi to grow. The rest of the genera found on the ungerminated seeds are not recorded as causing diseases in pines.

Although most of the treatments did not cause a statistically significant reduction in fungi, the presence of fungi on a seed is not a good predictor for whether the seed will germinate and produce healthy seedings (Himanen et al., 2013; Cram and Fraedrich 2009; Mittal and Wang 1993). Seedlings that germinate from high vigor seeds can outgrow some fungal contaminants (Kolotelo et al., 2001). In addition, the fungi that was identified was found on ungerminated seeds and could potentially be different than the fungi that appeared on germinated seeds. Future studies should grow seedlings in

potting media to determine what sanitation treatment is most effective at removing pathogens that cause seed and seedling mortality.

Conclusion

Propagation protocols are lacking for many native species, preventing their growth in nurseries and use in restoration and afforestation projects. Providing science-based protocols to nurseries that utilize materials that are easily procured on site and do not require specialized training is critical to success for local nurseries (Haase and Davis 2017). Based on the results of this study, *P. occidentalis* does not have dormant seeds and does not require any dormancy-breaking pretreatments. To reduce fungal infections, *P. occidentalis* seeds should be soaked in a 3% hydrogen peroxide solution for 1-hour followed by a 1-hour running water rinse. These propagation protocols will reduce seed and seedling loss to fungi, allowing for high-quality seedlings to be grown at affordable costs in nurseries in Haiti and the Dominican Republic.

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Table 2.1. List of sanitation treatments used in study. All bleach used had a concentration of 5.25% and all hydrogen peroxide had a 3% concentration.

Treatment	Description
1	2:3 bleach: water soak for 10 minutes, 12 hour running water rinse
2	1:5 bleach: water soak for 10 minutes, 12 hour running water rinse
3	11 hour rinse under running water, 2:3 bleach: water soak for 10 mins, 1 hour running water rinse
4	11 hour rinse under running water, 1:5 bleach: water soak for 10 mins, 1 hour running water rinse
5	2:3 bleach: water soak for 10 minutes, 30 minute running water rinse
6	1:5 bleach: water soak for 10 minutes, 30 minute running water rinse
7	1 hour hydrogen peroxide soak, 1 hour running water rinse
8	4 hour hydrogen peroxide soak, 1 hour running water rinse
9	12 hour hydrogen peroxide soak, 1 hour running water rinse
10	12 hour running water rinse
11	Control (No treatment or water soak)

Table 2.2. Results of the analysis for the effects of seed moisture content and cold stratification length on the odds of a seed germinating and the mean germination time (MGT) of *Pinus occidentalis* seeds.

Source of Variation	Odds of a seed germinating *P*	MGT *P*
Moisture content	0.9596	0.2319
Stratification length	0.2683	> 0.0001
Moisture content x stratification length	0.9417	0.6155

Table 2.3. The odds of a seed germinating (with 95% confidence intervals) and mean germination time (MGT) (with 95% confidence intervals) for each level of seed moisture content and length of cold stratification. Means and confidence intervals for each level of seed moisture content were averaged over all stratification lengths and means and confidence intervals for each cold stratification length were averaged over all seed moisture content levels. There was no significant difference in the odds of germination between the control and treatments for seed moisture content and cold stratification length. For MGT, letters A and a represent no difference with the treatment and control, while letters B and b represent a significant difference between the treatment and the control. Comparisons were made between the control and treatments using Dunnett's test ($\alpha = 0.05$).

	Odds of Germination (%)	MGT (days)
Moisture Content		
5%	71.5 (65.6, 76.8)	13.6 (13.1, 14.2) A
27%	74.1 (68.1, 79.3)	13.1 (12.6, 13.7) A
31%	74.3 (68.4, 79.4)	13.6 (13.0, 14.1) A
35% (control)	73.8 (68.0, 78.9)	12.9 (12.4, 13.5)
39%	73.5 (67.6, 78.6)	13.6 (13.1, 14.4) A
Stratification Length		
0 days (control)	74.1 (68.3, 79.2)	12.4 (11.9, 13.0)
7 days	70.5 (64.5, 75.8)	13.2 (12.6, 13.7) a
14 days	70.6 (64.6, 76.0)	13.4 (12.9, 14.0) b
21 days	73.0 (67.1, 78.2)	14.5 (13.9, 15.0) b
28 days	78.5 (72.9, 83.2)	13.4 (12.9, 13.9) b

Table 2.4. The odds of a seed germinating (with 95% confidence intervals), the mean germination time (MGT) (with 95% confidence intervals) and the percent of germinated seeds with mold (with 95% confidence intervals) for each sanitation treatment and the control. There was no significant difference in the odds of germination between the treatments and the control. For MGT and percent of germinated seeds with mold, letter A represents no difference between the treatment and control, while letter B represents a significant difference between the treatment and the control. Comparisons were made between the control and treatments using Dunnett's test ($\alpha = 0.05$).

Treatment	Odds of Germination (%)	MGT (days)	Germinated seeds with mold (%)
1	74 (64, 82)	13.9 (12.8, 15.0) A	2.7 (0.3, 22.7) A
2	68 (58, 75)	12.8 (11.7, 13.9) A	2.9 (0.3, 24.3) A
3	67 (57, 75)	14.6 (13.5, 15.6) A	4.5 (0.7, 24.6) A
4	77 (68, 84)	13.6 (12.6, 14.7) A	1.3 (0.04, 26.4) A
5	66 (56, 75)	16.3 (15.3, 17.4) B	25.8 (12.1, 46.7) A
6	76 (67, 83)	14.0 (13.0, 15.1) A	1.3 (0.05, 26.7) A
7	79 (70, 86)	13.9 (12.8, 15.0) A	3.8 (0.6, 21.5) B
8	80 (71, 87)	15.0 (14.0, 16.1) A	1.3 (0.05, 25.7) A
9	67 (57, 75)	12.8 (11.7, 13.9) A	1.5 (0.06, 29.4) A
10	68 (58, 76)	14.2 (13.1, 15.3) A	50.0 (31.1, 68.9) A
11 (control)	69 (59, 77)	13.1 (12.1, 14.2)	43.5 (25.7, 63.1)

Table 2.5: Fungi identified on ungerminated seeds from each sanitation treatment and the control.

	Treatment										
Genus	1	2	3	4	5	6	7	8	9	10	11 (control)
Alternaria[1]										X	
Fusarium[1]											X
Rhizoctonia[1]										X	
Botryosphaeria[2]		X	X								
Chaetomium[2]	X	X	X	X	X	X		X			X
Epicoccum[2]				X	X		X	X			
Ulocladium[2]	X	X		X	X						
Aspergillus[3]				X	X		X			X	X
Cladosporium[3]									X		
Penicillium[3]				X	X	X	X			X	X
Mortierella[4]										X	
Mucor[4]							X				
Phaeoacremonium[4]				X							
Pyrenochaeta[4]									X		
Rhizopus[4]									X		
Unknown	X	X	X	X	X		X	X	X		X

[1] Genera known to contain species that are known to cause seed or seedling death.

[2] Genera known to contain species that show weak pathogenicity that can cause death of damaged seeds or low-vigor seedlings.

[3] Saprophytic genera that feed on dead or decaying seed tissue and can cause seed death if seeds are stored incorrectly or if seeds are damaged. These genera do not harm seedlings.

[4] Not known to cause disease in conifer seeds or seedlings

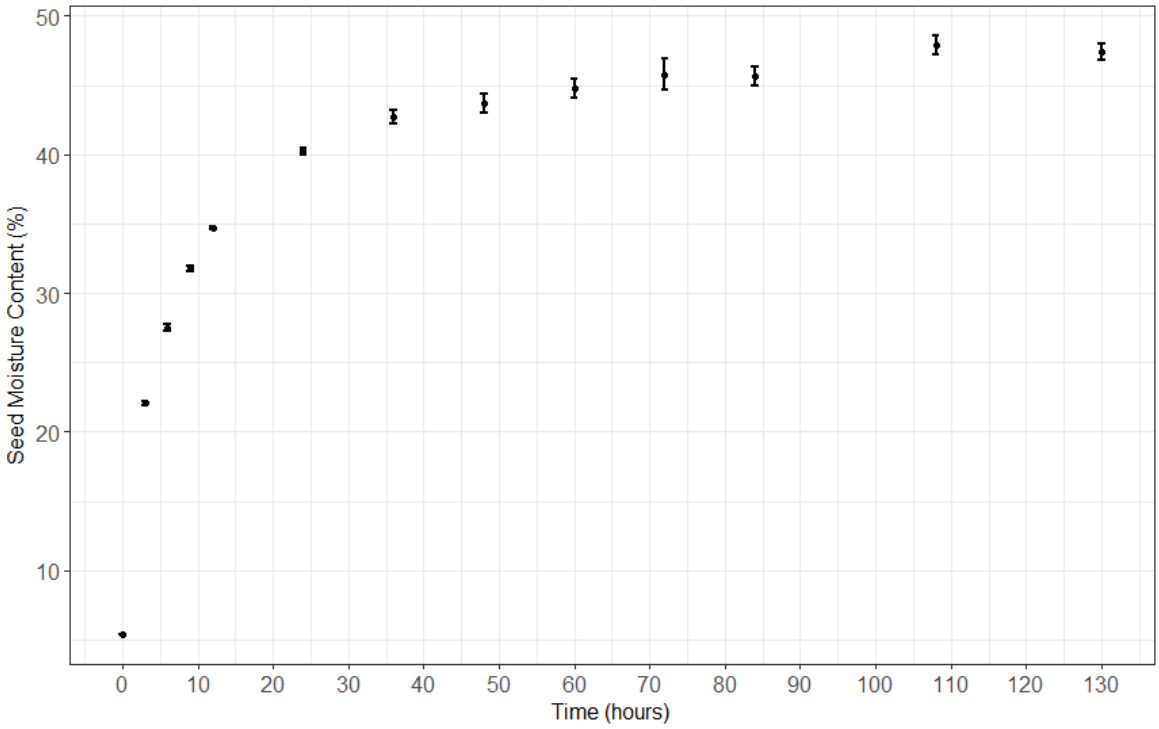

Figure 2.1. Water uptake curve (fresh weight basis) for *P. occidentalis.* Each point represents the mean (± s.e.) of 7 replicates.

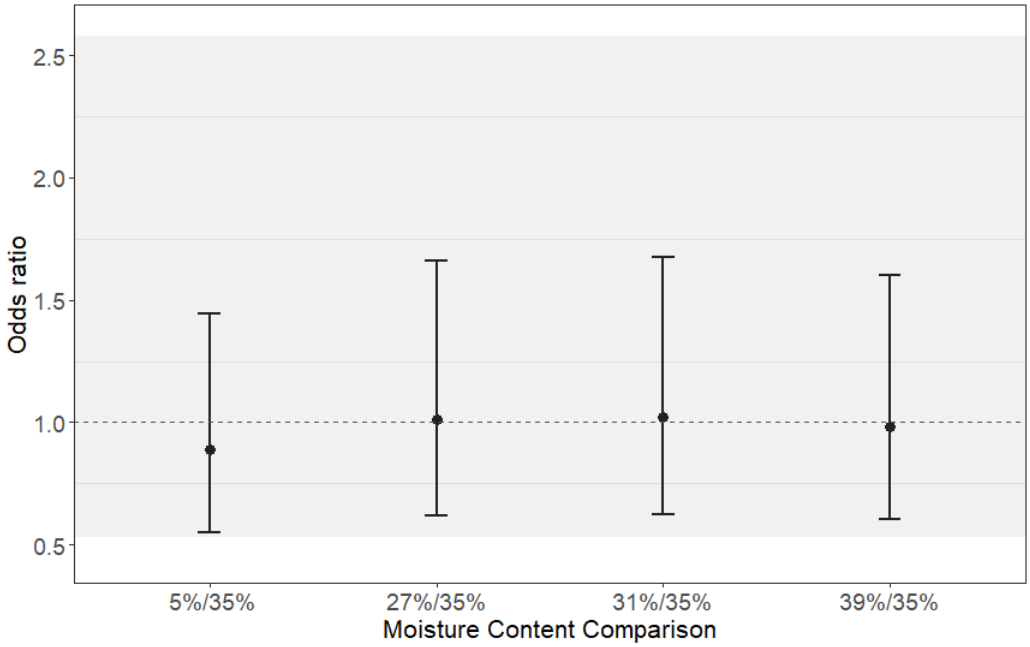

Figure 2.2. Ratios of estimated odds of germination for each seed moisture content when compared to the control (35%), with 95% confidence intervals. The dashed line at 1 indicates no statistical difference and the gray fill represents the area of no biological significance between 0.53 and 2.58.

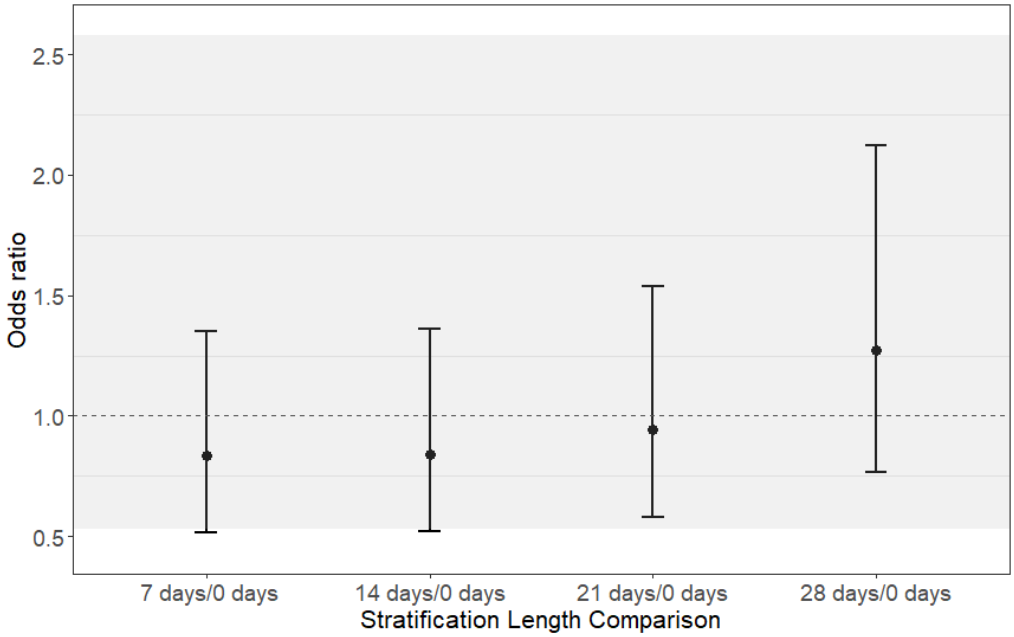

Figure 2.3. Ratios of estimated odds of germination for each cold stratification length when compared to the control (0 days), with 95% confidence intervals. The dashed line at 1 indicates no statistical difference and the gray fill represents the area of no biological significance between 0.53 and 2.58.

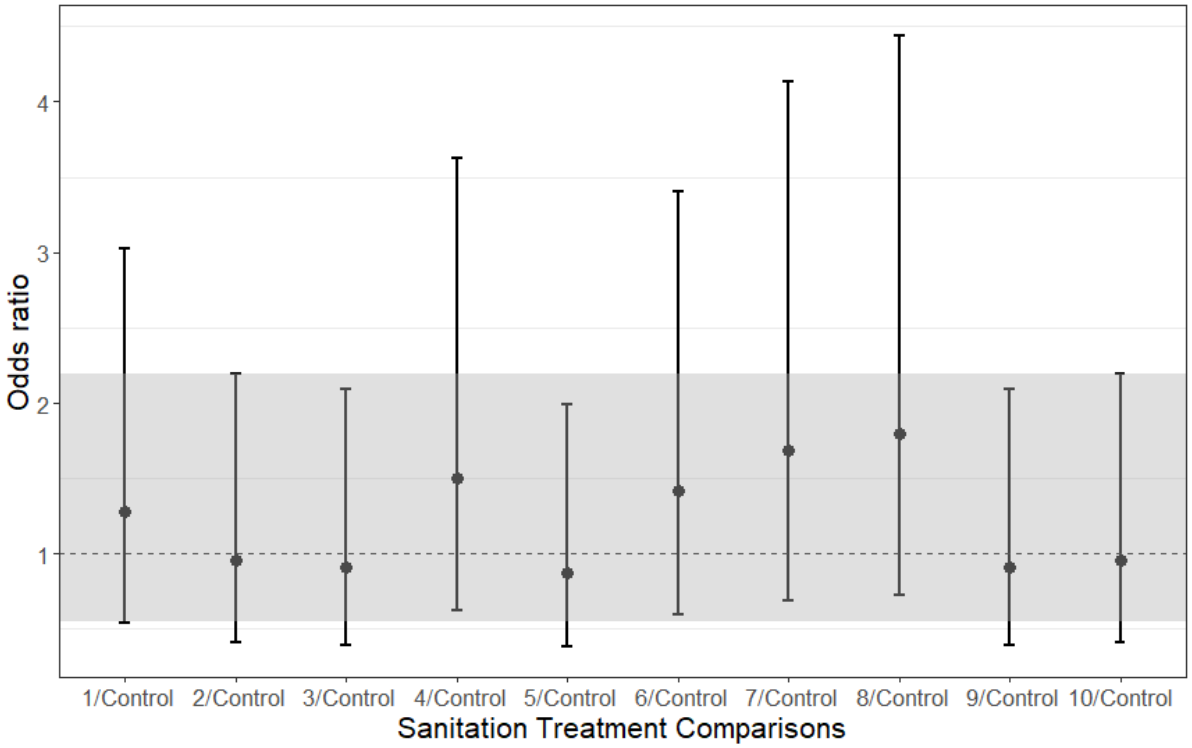

Figure 2.4. Ratios of estimated odds of germination for each sanitation treatment when compared to the control, with 95% confidence intervals. The dashed line at 1 indicates no statistical difference and the gray fill represents the area of no biological significance between 0.55 and 2.19. For treatments 2, 3, 5, 9 and 10 there is a potential of decreasing the odds of a seed germinating when compared to the control, and for treatments 1, 4, 6, 7, and 8, there is a possibility that those treatments could increase the odds of a seed germinating when compared to the control.

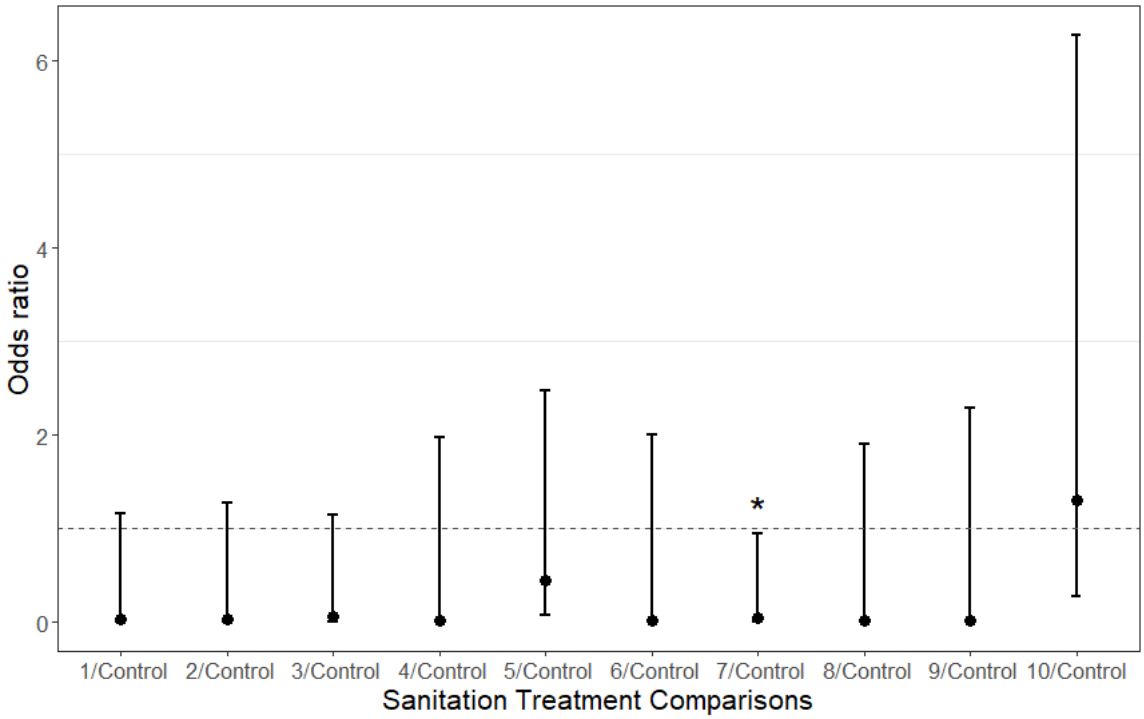

Figure 2.5. Ratio of the estimated odds of a germinated seed having mold when compared to the control, with 95% confidence intervals. The dashed line at 1 indicates no statistical difference. An asterisk (*) represents a significant difference from the control.

Chapter 3: The effects of preconditioning on survival, growth and water relations of *Pinus occidentalis* seedlings experiencing drought stress

Abstract

Water is often the most limiting resource on restoration and afforestation sites and determines seedling survival and growth. The practice of drought preconditioning, where seedlings are exposed to water stress while in the nursery, has proven successful in increasing the drought tolerance of some species. Hispaniolan pine (*Pinus occidentalis*) was subjected to three levels of drought preconditioning then to no drought, moderate drought and severe drought conditions simulating levels of moisture stress that could be experienced after planting. *Pinus occidentalis* grown under different preconditioning treatments had different morphology but there was no evidence of osmotic adjustment to drought stress. All preconditioning treatments had similar survival odds after the no drought and moderate drought stress treatments, but only moderate drought preconditioned seedlings had decent survival after experiencing severe drought stress. Seedlings grown with no preconditioning or moderate drought preconditioning have similar morphology when experiencing no drought or moderate drought stress. Seedlings from all preconditioning treatments that experience moderate drought stress had similar growth rates. The overall morphology of severe drought preconditioned seedlings remained stunted. Exposing *P. occidentalis* seedlings to moderate drought preconditioning in the nursery will increase survival on sites with extreme water stress without reducing growth rate.

Introduction

The most common stress that seedlings face after planting is water stress (Grossnickle and Folk 1993; Davis and Jacobs 2005). Restoration and afforestation sites have water cycles that differ from natural ecosystems. Disturbed sites tend to be hotter and dryer than natural ecosystems, leading to more soil evaporation and plant transpiration (Miller 1983). Recently planted seedlings are especially sensitive to soil moisture levels because their root systems have not become established in the soil. The roots of these seedlings have poor soil contact and are contained in a small area, limiting access to water and nutrients (Grossnickle 2005).

Although we have a view of tropical forests as constantly wet, drought does limit growth and survival in tropical forests during the dry season and many species are adapted to tolerate drought even as seedlings (Marod et al., 2002; Engelbrecht et al., 2005). Changing weather patterns may lead to prolonged periods of drought in Hispaniola, especially during the traditional start of the wet season (Karmalkar et al., 2013). Limited resources and geographical isolation may prevent restoration sites from being watered after planting, so seedlings must be capable of surviving and growing during drought experienced immediately after planting.

Drought affects seedlings by decreasing cell turgor and the rate of cell division, increasing solute concentrations, and reducing photosynthesis by closing stomata and limiting CO_2 uptake, which results in less photosynthates for root and shoot growth (Carlson and Miller 1990; Schuppler et al., 1998; Grossnickle 2005; Lawlor 2002). Seedlings have a variety of morphological and physiological mechanisms to deal with the effects of drought. Water stress triggers a complex series of responses in plants involving hundreds of genes and proteins (Chaves et al., 2003). It has been widely noted that there are certain morphological traits in conifer seedlings that increases the probability of surviving and growing

during droughty conditions. Shorter seedlings tend to have better survival and growth under drought conditions (Mexal and Landis 1990; McTague and Tinus 1996; Grossnickle 2012). A larger shoot system leads to more transpiration and oftentimes root systems cannot absorb enough water to maintain turgor in the seedling (Davis and Jacobs 2005; Grossnickle 2012). For loblolly pines, initial height was negatively correlated with seedling survival and height growth after two growing seasons on sites that experienced high drought stress (Tuttle et al., 1987). A larger root collar diameter (RCD) is seen as beneficial to seedlings experiencing drought stress. RCD is correlated to most other morphological measurements because of its importance to the movement of water and nutrients to the shoot system and photosynthates to the root system (Mexal and Landis 1990; Grossnickle 2012).

Some studies have suggested that a larger root system, as measured by root volume, is beneficial for survival and growth on droughty sites. The size of the root system at planting will determine initial water uptake for the seedling until new root growth commences (Carlson and Miller 1990). The size of the root system influences the root growth potential of the seedling, with larger root systems producing more new roots after planting (Carlson 1986). For ponderosa pine grown on droughty sites in the eastern Cascades, seedlings with the largest root volumes had higher survival rates, greater total height and larger annual height growth than seedlings with smaller root volumes (Rose et al., 1991; Rose et al., 1997). A large root volume may not be helpful in drought conditions if it is correlated to greater shoot system and high transpirational demand (Haase and Rose 1990). In addition, root volume does not provide an accurate estimate of root fibrosity, which determines roots system's absorptive ability (Thompson 1985; Haase 2008).

One mechanism that seedlings use to adjust to water stress is to increase root-to-shoot ratio (Kozlowski and Pallardy 2002). A larger root-to-shoot ratio results in less transpirational surface relative to water absorbing surface and is thought to be a good measure of how capable the root system is at

supplying water to the shoot system (Grossnickle and Folk 1993). In Scots pine, container seedlings grown under dry conditions had a 1.3-fold increase in root-to-shoot ratio when compared to seedlings grown under moist conditions (Moser et al., 2015). But there are conflicting views on how useful this trait is for container seedlings (McGilvray and Bartlett 1982; Thompson 1985; Bernier et al., 1995). The ability of a seedling to avoid drought stress after planting is most closely related to how quickly the seedling can grow new roots and there is no evidence that there is a relationship between root-to-shoot ratio and root growth potential (Bernier et al., 1995).

Physiological adaptations to drought allow seedlings to reduce the amount of water loss or continue metabolic processes with lower cellular water contents. Osmotic adjustment is a change in plant water potential by decreasing osmotic potential through increased solute concentration within cells (Gonzalez and Reigosa Roger 2001). Osmotic adjustment allows cells to maintain a particular relative water content (RWC) at decreased water potentials (Sinclair and Ludlow 1985). RWC of a cell is an important determinant of the ability to continue metabolic activities like photosynthesis and ATP production while under stress (Sinclair and Ludlow 1985; Lawlor 2002). RWC is defined as the proportion of water mass compared to the mass of the entire cell (Kirkham 2005). Maintaining an adequate RWC at turgor loss point (RWC_{TLP}) that allows for chemical reactions to continue is critical to maintaining metabolic functions in drought stressed plants (Lawlor 2002).

In southern pines, osmotic adjustment is likely due to changes in carbohydrate composition through the increased conversion of starch to sugar (Johnson et al., 1985). Two important measurements are osmotic potential at full turgor (Ψ_{FT}), when the cell is fully hydrated, and osmotic potential at turgor loss point (Ψ_{TLP}), when the cell permanently wilts and stops metabolic functions (Kirkham 2005). Ψ_{TLP} is seen as a direct quantifier of drought tolerance as it determines how negative plant water potential can go while still maintaining turgor and metabolic functions (Bartlett et al., 2014).

Osmotic adjustment causes changes in both Ψ_{TLP} and Ψ_{FT} (Bartlett et al., 2012). Osmotic adjustment as a mechanism to tolerate drought stress in conifers is common and is seen in loblolly pine (Hennessey and Dougherty 1984; Johnson et al., 1985), ponderosa pine (Anderson and Helms 1994), Monterey pine (De Diego et al., 2013) and Douglas-fir (Ritchie and Shula 1984). But some conifers, such as lodgepole pine (Stewart and Lieffers 1993), white spruce and jack pine (Marshall et al., 2000), do not use osmotic adjustment as a mechanism to cope with drought.

Modifications to cell wall elasticity (ε), or how easily the cell can deform without damage to membranes, can be either a drought avoidance or drought tolerance mechanism. Higher value of ε indicates a more rigid cell wall and allow cells to maintain values of RWC_{TLP} necessary for metabolic activity, even if the plant has a very low Ψ_{TLP} (Bartlett et al., 2012). Drought resistant varieties of white spruce have much higher ε than drought sensitive white spruce and were able to maintain cell turgor during lower cell relative water contents (Marshall and Dumbroff 1999). Lower value of ε implies greater cell elasticity and an increased ability to maintain turgor pressure when experiencing lower RWC (Grossnickle and Folk 1993; Clifford et al., 1998). Cells with more elastic tissues can maintain turgor longer than cells with more rigid cell walls (Kozlowski and Pallardy 2002). This mechanism is seen in maritime and lodgepole pines that have experienced severe drought stress (Fernandez et al., 1999; Stewart and Lieffers 1993).

Other mechanisms to avoid or tolerate drought are changes in stomatal conductance and gas exchange rates through leaf tissues. Decreasing transpiration rates and increasing CO_2 diffusion across mesophyll tissues have been seen in some loblolly pine families in response to drought preconditioning (Seiler and Johnson 1988). These two changes allowed CO_2 levels to remain high while reducing water loss, resulting in increased photosynthesis rates at lower needle water potentials. Similar reductions in transpiration rate have been recorded in Caribbean pine (Ali Abod and Sandi 1983). In contrast to these

two examples, drought preconditioned black spruce had higher transpiration rates than control seedlings and were able to maintain high photosynthesis rates during drought stress by keeping stomatal conductance high and CO_2 concentration high (Zine El Abidine et al., 1994b).

Nursery cultural practices can influence seedling survival and growth after planting (Grossnickle 2012) and there are nursery practices that can increase drought tolerance in seedlings (Landis 1989). The Target Plant Concept provides a framework for how to develop a seedling that can survive and grow in the specific environmental conditions found at the planting site (Dumroese et al., 2016). *Pinus occidentalis* had historically been found in a wide range of habitats, from 200m to 3200m in elevation and 800mm to more than 2300mm of precipitation a year (Darrow and Zanoni 1990). There will be a need to revegetate sites with varying moisture levels and nursery managers will need to know how *P. occidentalis* responds to water stress to grow plants that are specifically adapted to the water cycles likely experienced on the restoration or afforestation site.

Seedlings can be acclimated to drought by exposure to drought cycles in the nursery (Hennessey and Dougherty 1984). When experiencing water stress after planting, there is some evidence that seedlings that have had previous exposure to drought in the nursery have greater survival and growth than seedlings with no previous exposure (Kozlowski and Pallardy 2002). Drought preconditioning increased survival of Douglas-fir and lodgepole pine by 16% when exposed to water-stressed conditions after planting (van den Driessche 1991). Drought preconditioned stone pine seedlings had a lower osmotic potential at full turgor (Ψ_{FT}) and at turgor loss point (Ψ_{TLP}) than seedlings without preconditioning, allowing them to maintain metabolic activities under greater water stress (Deligoz and Gur 2015). Drought preconditioning is not an effective tool to increase the drought resistance of all species. Physiological modifications induced by drought preconditioning in the nursery can be short-lived and disappear before a seedling reaches the restoration or afforestation site or the species may

already have a high tolerance to drought that is not enhanced through preconditioning (Villar-Salvador et al., 2004; Royo et al., 2001).

Limited research has been done on the effect of drought on *P. occidentalis* seedling morphology and physiology. Hubbel (2015) found that *P. occidentalis* seedlings experiencing drought had lower predawn water potentials and rates of photosynthesis when compared to well-watered seedlings. No studies have investigated the effectiveness of drought preconditioning on *P. occidentalis*. This study examined the effect of drought on the morphology and water relations parameters of *P. occidentalis*. It also examined the effect of drought preconditioning on the survival, growth and water relations parameters of *P. occidentalis* experiencing different levels of water stress. It was hypothesized that drought preconditioned *P. occidentalis* seedlings would be smaller and show osmotic adjustment to drought stress relative to seedlings not preconditioned to drought. It was also hypothesized that drought preconditioned seedlings would have higher survival and faster growth rates when exposed to drought conditions relative to seedlings not preconditioned to drought.

Methods

Drought Preconditioning

A description of the seed lot used in this experiment can be found in the germination pre-treatment study (Chapter 2). This experiment was a completely randomized design, with trays assigned to one of three drought preconditioning treatments. Each tray represented an experimental unit containing 20 trees. Prior to sowing, seeds were cleaned in a 3:1 water:hydrogen peroxide solution for 5 minutes then soaked in aerated distilled water for 12 hours. Seeds were sown on July 3, 2017 into twenty-four trays of 10 in^3 Ray-Leach Conetainers (Stuewe and Sons, Tangent, OR, USA). Each tray contained 20 conetainers and each conetainer was sown with four seeds. Potting media was composed of 50% peat moss, 50% vermiculite with ½ tsp slow release fertilizer in each conetainer (Osmocote Smart Release 6 month Fertilizer 15-9-12 (N-P-K), The Scotts Company, Maryville, OH, USA). Seeds were covered with approximately 2mm of media and media was kept moist during germination. Pines were thinned to one tree per conetainer in August. On September 6, 2017 (three months after sowing), seedlings were large enough (height ≥ 2.5cm) to begin drought preconditioning.

Each tray was randomly assigned to a preconditioning treatment, which was defined by the container block weight when irrigation would occur as determined by the scientist method according to Dumroese et al. (2015). The control treatment (12 trays) was watered when trays reached 70% saturation weight; the moderate drought preconditioned treatment (8 trays) was watered when trays reached 30% saturation weight; and the severe drought preconditioned treatment (4 trays) was watered when trays reached 10% saturation weight. After each watering, a new saturation weight and target saturation weight for irrigation were determined. Tray location on the nursery bench was randomized

weekly. Seedlings were grown under the preconditioning drought treatments for slightly more than 3 months (9/6/2017-12/16/2017).

Morphological measurements were taken for all seedlings at the end of the preconditioning treatments. Seedlings that were eligible to be randomly selected for the next drought study were within 1cm of the mean height and 0.5mm of the mean diameter for each specific treatment to ensure that representative samples were chosen from each treatment. Five seedlings from each preconditioning treatment that met this requirement were randomly selected and height and root collar diameter were measured. Root volume for each sampled seedling was determined by the water displacement method (Burdett 1979). Root surface area was determined using the WinRHIZO Image Analysis software (Regents Instruments Inc., Quebec, QC, Canada). After all morphological measurements were taken, the shoot and root system of each seedling were dried separately at 63°C for 72 hours and used to determine the root-to-shoot ratio.

Three additional seedlings from each preconditioning treatment were randomly selected to construct pressure-volume curves using a pressure chamber (PMS Instrument Company, Albany, OR, USA). Seedlings were watered to saturation and placed in dark storage. The following day, the main stem of the seedling was cut and used for analysis. Seedling segments were weighed then immediately placed in the pressure chamber for measurement. Seedling segments transpired on the lab table between measurements. The dry weights of the segments were determined after placed in the oven at 63°C for 72 hours. Osmotic potential at turgor loss point (Ψ_{TLP}), relative water content at turgor loss point (RWC_{TLP}), relative water content at full turgor (RWC_{FT}), and bulk modulus of elasticity (ε) were estimated from the pressure-volume curves.

Simulated Drought Stress

This experiment is a randomized complete block design, with a factorial structure (three drought stress treatments x three preconditioning treatments). Each seedling represented an experimental unit and was considered independent of other seedlings. Seventeen seedlings from each preconditioning treatment, based on the selection criteria described above, were randomly assigned to one of three drought stress treatments, with a total of 51 seedlings in each drought stress treatment. After selection, seedling height and root collar diameter were measured and seedlings were transplanted into Tall-One Treepots (0.8 gallon) (Stuewe and Sons, Tangent, OR, USA). Hubbel (2015) reported that *P. occidentalis* is sensitive to transplant shock, so care was taken to ensure that root systems were undisturbed during transplanting. The seedlings were transplanted into the same media as the previous study (50% peat moss, 50% vermiculite) and were fertilized with slow release fertilizer with the manufacturer's recommended application rate of 3 tbsp per 2 gallons potting media (Osmocote Smart Release 6 month Fertilizer 15-9-12 (N-P-K), The Scotts Company, Maryville, OH, USA).

Seedlings in the no drought stress treatment were watered when they reached 70% saturation weight; seedlings in the moderate drought stress treatment were watered when they reached 40% saturation weight; and seedlings in the severe drought stress treatment were watered when they reached 10% saturation weight. The seedlings in the severe drought stress treatment never reached 10% saturation weight and were not watered during the drought study. The container block weight when irrigation would occur (target saturation weight) was again determined by the scientist method according to Dumroese et al. (2015). After each watering, a new saturation weight and target saturation weight when irrigation should occur was determined for each seedling. Seedlings were grown in a greenhouse with an average temperature of 25°C (minimum temperature of 10°C, maximum

temperature of 46°C). Seedlings were grown under natural light with supplemental LED lighting [70 µmol m^{-2} s^{-1} mean photosynthetic photon flux density (PPFD)] to provide a 12-hour photoperiod. Seedling location on nursery benches was randomized once a week. Seedlings were grown under the drought stress treatments for 3 months (12/17/2017-3/18/2018).

Predawn water potential (Ψ_{pd}) measurements were taken when seedlings reached their target saturation weight using a pressure chamber (PMS Instrument Company, Albany, OR, USA). Due to the small size of the seedlings, they were destructively sampled and the main stem of the seedling was used for the measurements. Each treatment reached its target saturation weight at different rates, so the measurements were taken on different dates for each drought treatment. The no drought stress treatment had Ψ_{pd} measurements taken in January and March (after their second and eighth waterings) while the moderate drought stress treatment had Ψ_{pd} measurements taken in February and March (after their first and second waterings). There were not enough living trees to take measurements for the severe drought stress treatment.

At the end of the drought study (3/18/2018), survival data for all seedlings was recorded. Morphological measurements for each preconditioning and drought treatment combination were taken at the end of the drought study. Five seedlings from each treatment combination were randomly selected and height and root collar diameter were measured, and height and root collar diameter growth for each seedling was determined. Root volume, root surface area and root-to-shoot ratio were determined using the same methods described in the preconditioning study. In the severe drought treatment, high mortality prevented measurements being taken on the control and severe drought preconditioned seedlings. Pressure-volume curves were constructed for three seedlings, if available, for each preconditioning and drought treatment combination. Pressure-volume curves were constructed using the same method described in the previous study.

Statistical Analysis

All analyses were done with R (Version 3.4.3, The R Foundation for Statistical Computing, 2017). All effects of interest were estimated from the corresponding models and differences between treatments for each parameter were analyzed using one-way ANOVAs and comparisons were made using Tukey HSD ($\alpha = 0.05$).

A generalized linear model using a quasi-binomial distribution with the logit link was used to test the effect of preconditioning treatment on the odds of a seedling surviving until the end of treatment. Linear models were used to test differences in morphological measurements (height, height growth, RCD, RCD growth, root volume, root surface area, and root-to-shoot ratio) between preconditioning treatments. If the assumptions of normality of residuals and equal variance among preconditioning treatments were not met, parameters were log-transformed. Linear models using generalized least squares were used to test the differences in parameters estimated from pressure-volume curves.

For analysis of the simulated drought stress study, the effect of the preconditioning treatments on the survival, morphological measurements and water relations parameters of seedlings during each drought stress treatment were analyzed independent of the other drought stress treatments. There was no comparison among preconditioning treatments that experienced different drought stress treatments. Generalized linear models using a binomial distribution with the logit link were used to test the effect of preconditioning treatment on the odds of surviving to the end of the drought study. Linear models using generalized least squares were used to test the differences in morphology and water relations parameters between preconditioning treatments for each drought stress treatment. Linear models were used to test the differences in Ψ_{pd} between preconditioning treatments for each drought stress treatment.

Results

Drought Preconditioning

There was a difference in the odds of survival among the three preconditioning treatments ($p = 0.006$) (Table 3.1). Seedlings in the severe drought preconditioned treatment had lower survival odds (0.88) than the control (0.98) or the moderate drought preconditioned treatment (0.99) (Figure 3.1).

All morphological parameters were influenced by preconditioning (Table 3.1). There was a difference in height among preconditioning treatments ($p < 0.0001$) (Figure 3.2). When compared to control seedlings, moderate drought preconditioned seedlings were 28% smaller and severe drought preconditioned seedlings were 56% smaller. Severe drought preconditioned seedlings were 39% smaller than moderate drought preconditioned seedlings. Preconditioning also had a significant effect on RCD ($p < 0.0001$) (Figure 3.3), with control seedlings having a mean RCD that was 45% larger than moderate drought preconditioned seedlings and 85% larger than severe drought preconditioned seedlings. The mean RCD of moderate drought preconditioned seedlings was 27% larger than severe drought preconditioned seedlings. Root volume was affected by preconditioning ($p < 0.0001$) (Figure 3.4): the mean root volume of control seedlings was 55% and 82% larger than that of moderate and severe drought preconditioned seedlings, respectively. Root surface area was also affected by preconditioning ($p < 0.0001$) (Figure 3.5): the mean root surface area of control seedlings was 60% and 80% larger than that of moderate and severe drought preconditioned seedlings, respectively. Root to shoot ratio was affected by preconditioning ($p = 0.02$) (Figure 3.6), with seedlings in the severe drought preconditioned treatment having a mean ratio 40% lower than those in the moderate drought preconditioned treatment.

The only water relations parameter that differed among preconditioning treatments was the bulk modulus of elasticity (ε) ($p = 0.048$) (Figure 3.7). Seedlings in the severe drought preconditioned treatment had an ε that was 34% smaller than that of seedlings in the moderate drought preconditioned treatment. The severe drought preconditioned treatment had an ε that was 22% smaller than that of control seedlings, but the difference was not statistically significant ($p = 0.14$). Osmotic potential at turgor loss point (Ψ_{TLP}) was close to being statistically different among treatments ($p = 0.07$), while RWC_{TLP} and RWC_{FT} did not differ among treatments ($p = 0.38$ and $p = 0.69$, respectively) (Table 3.1). Table 3.3 provides the estimated means and 95% confidence intervals for all morphological and water relations parameters for each treatment.

Simulated Drought Stress

In the no drought stress treatment, there was no statistical evidence of a difference in seedling survival among preconditioning treatments ($p = 0.12$) (Table 3.2) (Figure 3.8). Significant differences existed among preconditioning treatments for all morphological parameters (all p values < 0.0001) (Table 3.2) (Figures 3.10 to 3.16). Seedlings from the severe drought preconditioned treatment were significantly smaller than control and moderate drought preconditioned seedlings for every morphological trait (Table 3.4). Although moderate drought preconditioned seedlings started the simulated drought stress study significantly smaller than control seedlings, by the end of the experiment there was no statistically significant difference between the two preconditioning treatments in any morphological trait except RCD (Table 3.4). There was statistical evidence that mean height growth differed among drought preconditioning treatments ($p = 0.038$) (Figure 3.11), though no individual comparison between treatments was statistically significant. There was statistical evidence that mean RCD growth differed among drought preconditioning treatments ($p < 0.0001$) (Figure 3.13). Mean RCD

growth for control seedlings was 23% larger than for moderate drought preconditioned seedlings and 66% larger than for severe drought preconditioned seedlings. Mean RCD growth for moderate drought preconditioned seedlings was 56% larger than that for severe drought preconditioned seedlings. There was no statistical evidence of a difference in mean predawn water potentials (Ψ_{pd}) among drought preconditioning treatments at the end of the second complete drydown (January 2018) ($p = 0.69$) or after the eighth complete drydown (March 2018) ($p = 0.44$). No significant differences existed among the preconditioning treatments for Ψ_{TLP} ($p = 0.97$), RWC_{TLP} ($p = 0.23$), RWC_{FT} ($p = 0.21$), or ε ($p = 0.34$) (Tables 3.2 and 3.4).

In the moderate drought stress treatment, there was no statistical evidence of a difference in seedling survival among preconditioning treatments ($p = 0.29$) (Figure 3.8). Significant differences existed among drought preconditioning treatments with respect to height ($p < 0.0001$) (Figure 3.10), RCD ($p = 0.004$) (Figure 3.12), root volume ($p < 0.001$) (Figure 3.14), and root surface area ($p < 0.001$) (Figure 3.15) (Tables 3.2 and 3.5). The difference in root-to-shoot ratios among preconditioning treatments was close to statistical significance ($p = 0.08$) (Figure 3.16). When exposed to moderate drought stress, control and moderate drought preconditioned seedlings presented similar morphologies with the exception of height, where moderate drought preconditioned seedlings were 28% smaller than control seedlings. Severe drought preconditioned seedlings were significantly smaller than control seedlings for every morphological parameter except root-to-shoot ratio. When compared to moderate drought preconditioned seedlings, severe drought preconditioned seedlings had significantly smaller mean height, RCD, and root volume, but had similar root surface area and root-to-shoot ratios. There was no statistical evidence that mean height growth or RCD growth differed among drought preconditioning treatments ($p = 0.97$ and $p = 0.18$, respectively) (Figures 3.11 and 3.13). There was no statistical evidence of a difference in mean Ψ_{pd} between preconditioning treatments at the end of the

first complete drydown (February 2018) ($p = 0.17$), but there was statistical evidence of a difference at the end of the second complete drydown (March 2018) ($p = 0.03$). The mean Ψ_{pd} for severe drought preconditioned seedlings was 0.18 MPa lower than the mean predawn water potential for moderate drought preconditioned seedlings. No significant differences existed between moderate and severe drought preconditioned seedlings for Ψ_{TLP} ($p = 0.32$), RWC_{TLP} ($p = 0.27$), RWC_{FT} ($p = 0.91$), or ε ($p = 0.85$) (Tables 3.2 and 3.5). There were not enough surviving control seedlings to measure water relations parameters.

For the severe drought stress treatment, there was statistical evidence for a difference in seedling survival odds among drought preconditioning treatments ($p = 0.004$) (Table 3.2) (Figure 3.9). The odds of a control seedling surviving were 0.06 (95% CI: 0.0, 0.32), while the odds of a moderate drought preconditioned seedling surviving were 0.35 (95% CI: 0.17, 0.60). No severe drought preconditioned seedlings survived. No morphological or water relations comparisons were made because of the lack of surviving seedlings in the control and severe drought preconditioned treatments.

Discussion

Drought Preconditioning

Drought preconditioning resulted in differences in most morphological traits and in the bulk modulus of elasticity (ε) of *Pinus occidentalis* seedlings. These results are not surprising. It is well documented that imposing drought stress to seedlings leads to a decrease in morphological parameters (Seiler and Johnson 1984; Royo et al., 2001; Deligoz and Gur 2015). The only morphological trait that did not differ among all drought treatments was root-to-shoot ratio, where the only statistically significant difference found was between moderate drought and severe drought preconditioned seedlings (Figure 3.6). Although moderate drought preconditioned seedlings had a slightly larger ratio than control seedlings, the results were not statistically significant. Some studies suggest that increasing root-to-shoot ratio is one mechanism that seedlings use to adjust to water stress, but there are conflicting results in the literature. Root-to-shoot ratio was increased by drought preconditioning in Scots pine (Moser et al., 2015), was unaffected by drought preconditioning in ponderosa pine (Stewart and Lieffers 1993) and decreased in drought preconditioned loblolly pine seedlings (Seiler and Johnson 1988). *Pinus occidentalis* does not show a consistent root-to-shoot ratio pattern when comparing drought preconditioning treatments. Moderate drought preconditioned seedlings could have adjusted allocation of resources between root and shoot systems to compensate for the reduced access to water when compared to control seedlings. The severe drought preconditioning treatment may have been too harsh to allow for resources to be shifted from shoot to root system.

Osmotic adjustment did not occur in *P. occidentalis*, although large variation within treatments may have obscured differences. If *P. occidentalis* uses osmotic adjustment as a drought tolerance mechanism, both the moderate and severe drought preconditioned seedlings should have experienced

sufficient drought stress to induce responses in water relations that would lead to drought hardening (Royo et al., 2001). In seedlings that osmotically adjust there is a lowering of osmotic potential and relative water content at the turgor loss point and at full turgor (Deligoz and Gur 2015; Fernandez et al., 1999; Johnson et al., 1985). A biologically significant adjustment to drought stress would be a decrease of at least 0.3 MPa (Johnson et al., 1985). Although the differences between the means of Ψ_{TLP} between treatments are below 0.3 MPa, all confidence intervals for the differences contain a decrease of 0.3 MPa. Future research with larger sample sizes is needed to clarify whether *P. occidentalis* uses osmotic adjustment as a drought tolerance mechanism.

A change in the bulk modulus of elasticity (ε), either through an increase or decrease in ε, is a physiological modification to drought stress (Bartlett et al., 2012). The severe drought preconditioned seedlings saw a 22% and 35% decrease when compared to control and moderate drought preconditioned seedlings, respectively. Cells with lower values of ε have a greater ability to change shape and will reach equilibrium turgor pressure with lower osmotic potential (Johnson et al., 1985; Tognetti et al., 2002). For plants that decrease ε as a drought tolerance mechanism, there is commonly a corresponding increase in RWC_{TLP} (Bartlett et al., 2012). This contrasts to what was seen in the severe drought preconditioned seedlings, which had a slightly lower RWC_{TLP} than control and moderate drought preconditioned seedlings (2.8% and 4.5%, respectively). The change in ε seen in severely drought preconditioned seedlings may not have been large enough to be biologically important or the difference between treatments may have been caused by small sample size.

Simulated Drought Stress

Preconditioning only affected survival in the severe drought treatment. When experiencing no or moderate drought, *P. occidentalis* appears to have a high survival rate irrespective of seedling morphology. Although morphology can affect survival, these results are not surprising. First year survival of loblolly and Aleppo pine under non-drought conditions are not affected by drought preconditioning (Seiler and Johnson 1984; Royo et al., 2001). Seedling mortality is correlated to precipitation rather than morphology in Aleppo pine (Royo et al., 2001), and the results of this study support that conclusion for *P. occidentalis* as well.

For the severe drought treatment, only the moderate drought preconditioning treatment had any significant survival. This may have been due to a higher initial root-to-shoot ratio, which is linked to survival in some conifers. Loblolly pine seedlings with twice the root-to-shoot ratio had 33% better survival under drought conditions than seedlings with lower root-to-shoot ratios (Boyer and Smith 1987). Larger seedlings, such as the control seedlings, tend to have poor survival on sites experiencing severe drought (Tuttle et al., 1987). The larger root system of control seedlings was not an accurate indicator of survival in the severe drought treatment. A larger RCD, as seen in control seedlings, is optimal for droughty sites (Mexal and Landis 1990) but the size of the shoot system in relation to root system may have been too great to allow for survival. The severe drought preconditioning treatment may have been too extreme and produced weak seedlings that did not have enough reserves to survive another 3 months of drought, even though they were able to survive in the no drought and moderate drought treatments.

Preconditioning treatment had no effect on height growth in either drought stress treatment where morphology could be measured (Figure 3.11). Preconditioning treatment affected RCD growth

when seedlings experienced no drought stress, but all preconditioning treatments had similar RCD growth when experiencing moderate drought stress (Figure 3.13). Size differences between moderate drought preconditioned seedlings and control seedlings lessened under both drought stress levels. Although larger seedlings tend to have higher growth rates in well-watered sites and lower growth rates on droughty sites than smaller seedlings, these trends are not consistent. In loblolly pines, the initial heights of seedlings between 7 and 13 inches did not affect total height after 1.5 years in field (Barnett and Brissette 1986). In slash pine, all drought preconditioning treatments (except for the most extreme treatment) had the same relative height growth after experiencing no drought upon planting (Johnson et al., 1985).

Control and moderate drought preconditioned seedlings had similar growth rates and morphology after experiencing no and moderate drought stress. There appears to be a wide range of *P. occidentalis* morphology that can be planted onto sites with varying levels of moisture stress without compromising future growth rates or overall size. The overall morphology for severe drought preconditioned seedlings remained stunted at the end of all drought stress experiments. This drought preconditioning treatment may be too severe and reduced growth potential in seedlings, even when subsequently exposed to well-watered conditions.

Pre-dawn water potentials (Ψ_{pd}) did not vary among preconditioning treatments when seedlings were well-watered but did vary when exposed to moderate drought stress. When seedlings have abundant water available to them, drought adaptations may not be expressed (Zine El Abidine et al., 1994a). Under well-watered conditions, no difference in Ψ_{pd} was found among different levels of drought preconditioned seedlings of black spruce and Aleppo pine, though differences were apparent when exposed to drought stress (Lamhamedi et al., 1996; Villar-Salvador et al., 1999).

In the moderate drought treatment, there was one significant difference in Ψ_{pd} after the second drydown, with moderate drought preconditioned seedling having a higher Ψ_{pd} than severe drought preconditioned seedlings. This would suggest that moderate drought preconditioned seedlings were less drought stressed than severe drought preconditioning seedlings. Mild differences in water stress should manifest in differences in growth (Myers 1988), but no differences were seen in height and RCD growth between the moderate and severe drought preconditioned seedlings and root surface area was similar by the end of experiment. It is possible that the difference in Ψ_{pd} was not biologically significant and did not affect seedling physiology or growth.

Under no drought stress, there were no differences in water relations parameters among the preconditioning treatments, similar to what was seen immediately following preconditioning (Tables 3.4 and 3.5). The bulk modulus of elasticity (ε) no longer showed differences among preconditioning treatments. Seasonal changes in cell elasticity are common in plants (Tognetti et al., 2002) and severe drought preconditioned seedlings could have modified cell wall elasticity when exposed to less water stress to have a similar value as other preconditioning treatments.

There are physiological adaptations other than osmotic adjustment that *P. occidentalis* could employ to survive and grow during drought. Modifications to stomatal conductance, rates of CO_2 diffusion and photosynthesis rates could occur without any changes to water relation parameters (Bartlett et al., 2012; Villar-Salvador et al., 1999; Zine El Abidine et al., 1994b; Seiler and Johnson 1988). There is evidence that photosynthesis rates are affected by drought conditions in *P. occidentalis* (Hubbel 2015). Future research should examine transpiration and photosynthesis rates in drought preconditioned *P. occidentalis* seedlings.

Conclusion

Based on the results of this study, *P. occidentalis* seedlings should be grown under the moderate drought preconditioning treatment. Seedlings grown under this nursery treatment have similar morphology and growth as control seedlings when experiencing no drought or moderate drought but have increased survival under extreme drought conditions. The severe drought preconditioning treatment is too harsh and results in lower quality seedlings. *Pinus occidentalis* does not appear to use osmotic adjustment but perhaps adjusts other aspects of physiology such as stomatal conductance to adapt to drought conditions.

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Table 3.1 Results of the analysis for the effect of preconditioning on morphology and physiology of *Pinus occidentalis* seedlings.

Source of Variation	Preconditioning *P*
Survival	0.0062
Height	< 0.0001
RCD	< 0.0001
Root volume	< 0.0001
Root surface area	< 0.0001
R:S	0.0244
Ψ_{TLP}	0.0680
RWC_{TLP}	0.6931
RWC_{FT}	0.9738
ε	0.0482

Table 3.2 Results of the analysis for the effect of preconditioning on morphology and physiology of *Pinus occidentalis* seedlings that experienced different levels of drought stress after planting.

		Preconditioning *P*	
Source of Variation	No Drought	Moderate Drought	Severe Drought
Survival	0.1243	0.2852	0.0039
Height	< 0.0001	< 0.0001	—
Height growth	0.0378	0.9650	—
RCD	< 0.0001	0.0040	—
RCD growth	< 0.0001	0.1786	—
Root volume	< 0.0001	0.0003	—
Root surface area	< 0.0001	< 0.0001	—
R:S	< 0.0001	0.0769	—
Ψ_{pd} (first measurement)	0.6938	0.1669	—
Ψ_{pd} (second measurement)	0.4372	0.0308	—
Ψ_{TLP}	0.9712	0.3188	—
RWC_{TLP}	0.2283	0.2680	—
RWC_{FT}	0.2143	0.9119	—
ε	0.3386	0.8459	—

Table 3.3. Means (with 95% confidence intervals) for morphological characteristics and water relation parameters for *Pinus occidentalis* seedlings after drought preconditioning. All parameters were estimated from their respective models. Root volume and root surface area are back-transformed means with 95% confidence intervals. Different letters indicate significant differences ($p < 0.05$) among treatments.

Seedling Trait	No Preconditioning	Moderate Drought Preconditioning	Severe Drought Preconditioning
Survival odds	0.98 (0.95, 1.0) a	0.99 (0.93, 1.0) a	0.88 (0.75, 0.94) b
Height (cm)	10.8 (10.0, 11.6) a	7.8 (7.0, 8.6) b	4.8 (4.0, 5.6) c
RCD (mm)	2.38 (2.19, 2.58) a	1.64 (1.45, 1.84) b	1.29 (1.10, 1.48) c
Root volume (cm^3)	2.2 (1.7, 2.8) a	1.0 (0.8, 1.3) b	0.4 (0.3, 0.5) c
Root surface area (cm^2)	83.74 (58.86, 119.12) a	33.31 (23.41, 47.38) b	16.41 (11.53, 23.34) c
R:S	0.65 (0.48, 0.81) ab	0.86 (0.70, 1.03) a	0.52 (0.36, 0.69) b
Ψ_{TLP} (MPa)	-1.05 (-1.14, -0.96) a	-1.12 (-1.50, -0.74) a	-1.22 (-1.33, -1.11) a
RWC_{TLP}	80.8 (77.1, 84.5) a	82.2 (74.6, 89.8) a	78.5 (75.5, 81.5) a
RWC_{FT}	91.4 (88.3, 94.5) a	89.1 (83.3, 94.9) a	91.1 (87.7, 94.4) a
ε (MPa)	4.02 (3.56, 4.48) ab	4.75 (3.88, 5.63) a	3.15 (2.32, 3.98) b

Table 3.4. Means (with 95% confidence intervals) for morphological characteristics and water relation parameters after the no drought stress treatment for *P. occidentalis* seedlings that experienced different drought preconditioning treatments. All parameters were estimated from their respective models. Different letters indicate significant differences ($p < 0.05$) among treatments.

Seedling Trait	No Preconditioning	Moderate Drought Preconditioning	Severe Drought Preconditioning
Survival odds	1.0* a	1.0* a	0.85 (0.54, 0.96) a
Height (cm)	16.7 (13.7, 19.7) a	12.8 (10.6, 15.1) a	7.8 (7.3, 8.3) b
Height growth (cm)	5.7 (3.7, 7.6) a	5.1 (3.2, 7.0) a	3.5 (3.3, 3.8) a
RCD (mm)	4.99 (4.50, 5.49) a	4.07 (3.84, 4.31) b	2.52 (2.33, 2.72) c
RCD growth (mm)	2.81 (2.47, 3.14) a	2.16 (1.96, 2.36) b	0.95 (0.88, 1.03) c
Root volume (cm^3)	5.4 (3.8, 6.9) a	3.5 (2.3, 4.7) a	0.7 (0.4, 1.1) b
Root surface area (cm^2)	131.66 (87.54, 175.79) a	87.81 (58.88, 116.74) a	23.53 (18.49, 28.57) b
R:S	0.25 (0.22, 0.27) a	0.21 (0.17, 0.24) a	0.14 (0.11, 0.18) b
Ψ_{pd} (Jan 2018)	-0.55 (-0.66, -0.45) a	-0.56 (-0.67, -0.46) a	-0.61 (-0.71, -0.50) a
Ψ_{pd} (March 2018)	-0.42 (-0.52, -0.32) a	-0.46 (-0.56, -0.36) a	-0.50 (-0.60, -0.40) a
Ψ_{TLP} (MPa)	-1.20 (-1.36, -1.02) a	-1.18 (-1.24, -1.12) a	-1.20 (-1.51, -0.88) a
RWC_{TLP}	82.5 (77.0, 88.0) a	80.7 (80.0, 81.6) a	76.6 (70.8, 82.3) a
RWC_{FT}	92.0 (89.6, 94.3) a	90.5 (89.9, 91.1) a	87.8 (83.9, 92.7) a
ε (MPa)	5.74 (5.04, 6.44) a	5.31 (4.94, 5.68) a	4.51 (2.12, 6.90) a

* No confidence interval could be determined.

Table 3.5. Means (with 95% confidence intervals) for morphological characteristics and water relation parameters after the moderate drought stress treatment for *P. occidentalis* seedlings that experienced different drought preconditioning treatments. All parameters were estimated from their respective models. Different letters indicate significant differences ($p < 0.05$) among treatments.

Seedling Trait	No Preconditioning	Moderate Drought Preconditioning	Severe Drought Preconditioning
Survival odds	0.36 (0.16, 0.62) a	0.64 (0.38, 0.84) a	0.43 (0.21, 0.68) a
Height (cm)	13.0 (11.1, 14.9) a	9.3 (8.5, 10.1) b	6.6 (5.8, 7.3) c
Height growth (cm)	2.1 (-0.1, 4.3) a	2.2 (1.5, 2.9) a	2.1 (1.4, 2.8) a
RCD (mm)	3.39 (2.76, 4.02) a	2.92 (2.51, 3.34) a	2.24 (1.97, 2.52) b
RCD growth (mm)	1.24 (0.53, 1.96) a	1.06 (0.80, 1.31) a	0.75 (0.48, 1.03) a
Root volume (cm^3)	1.9 (1.3, 2.4) a	1.5 (0.8, 2.3) a	0.5 (0.3, 0.6) b
Root surface area (cm^2)	67.34 (56.18, 78.53) a	56.39 (25.28, 87.51) ab	22.55 (15.41, 29.68) b
R:S	0.33 (0.23, 0.42) a	0.28 (0.21, 0.36) a	0.23 (0.21, 0.25) a
Ψ_{pd} (Feb 2018)	-1.57 (-1.84, -1.30) a	-1.48 (-1.74, -1.21) a	-1.24 (-1.50, -0.97) a
Ψ_{pd} (March 2018)	-0.60 (-0.69, -0.51) ab	-0.48 (-0.57, -0.40) a	-0.66 (-0.75, -0.58) b
Ψ_{TLP} (MPa)	NA	-1.29 (-1.91, -0.67) a	-1.02 (-1.64, -0.40) a
RWC_{TLP}	NA	76.3 (64.5, 88.0) a	82.1 (70.4, 93.9) a
RWC_{FT}	NA	88.2 (76.3, 100.0) a	87.7 (75.8, 99.7) a
ε (MPa)	NA	5.04 (4.09, 5.98) a	5.38 (0, 12.02) a

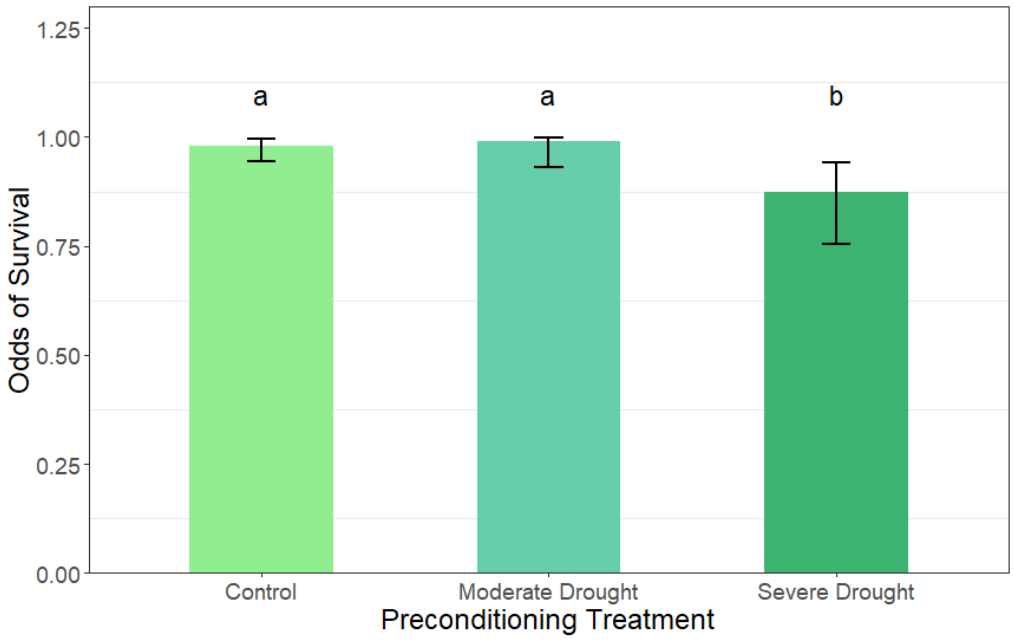

Figure 3.1. Mean odds of survival (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

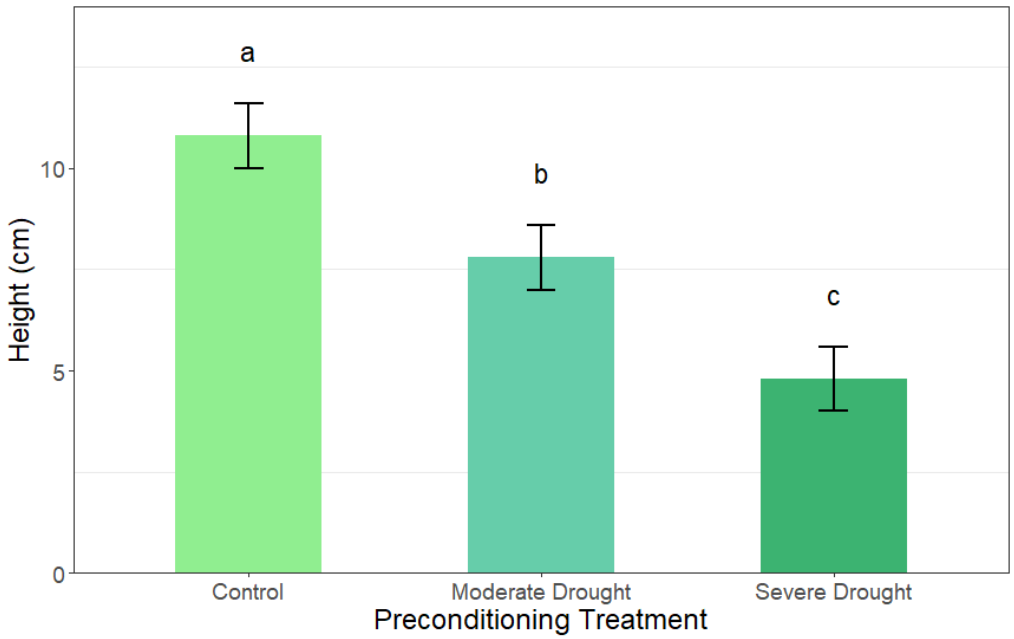

Figure 3.2. Mean height (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

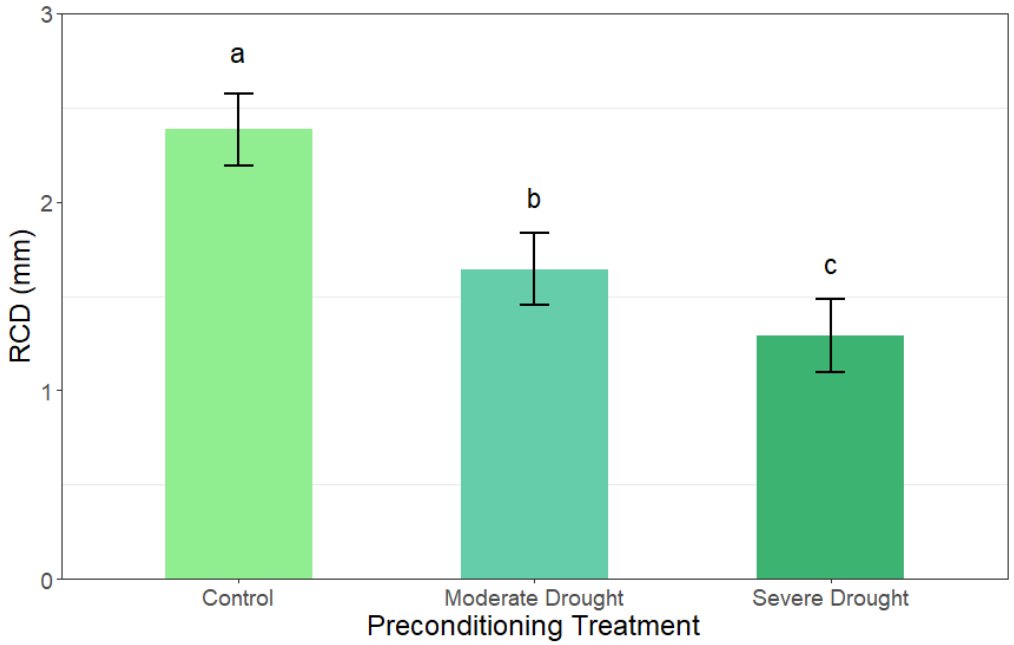

Figure 3.3. Mean root collar diameter (RCD) (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

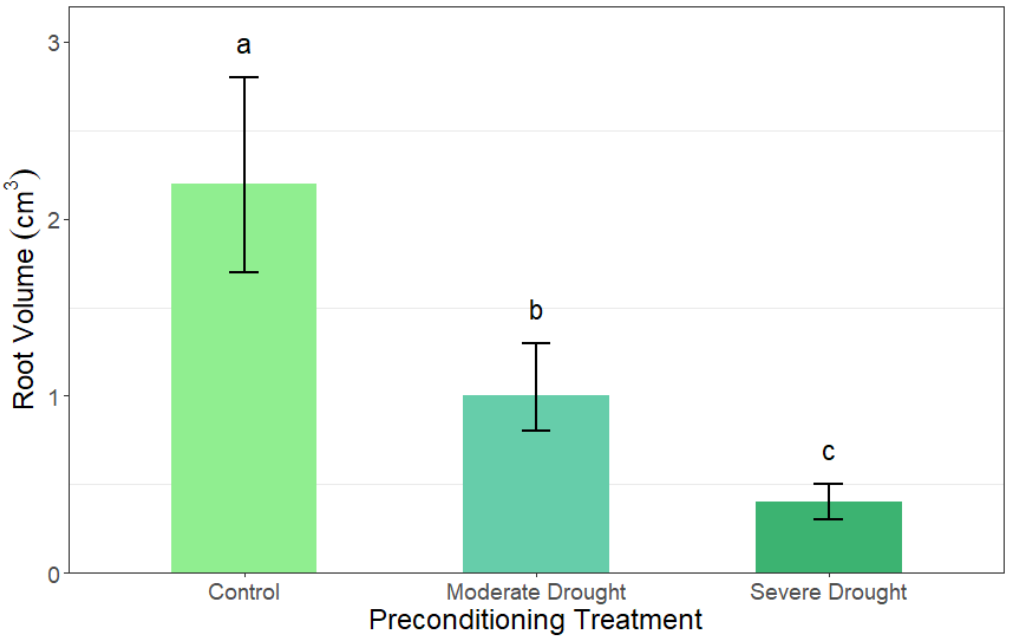

Figure 3.4. Mean back-transformed root volume (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

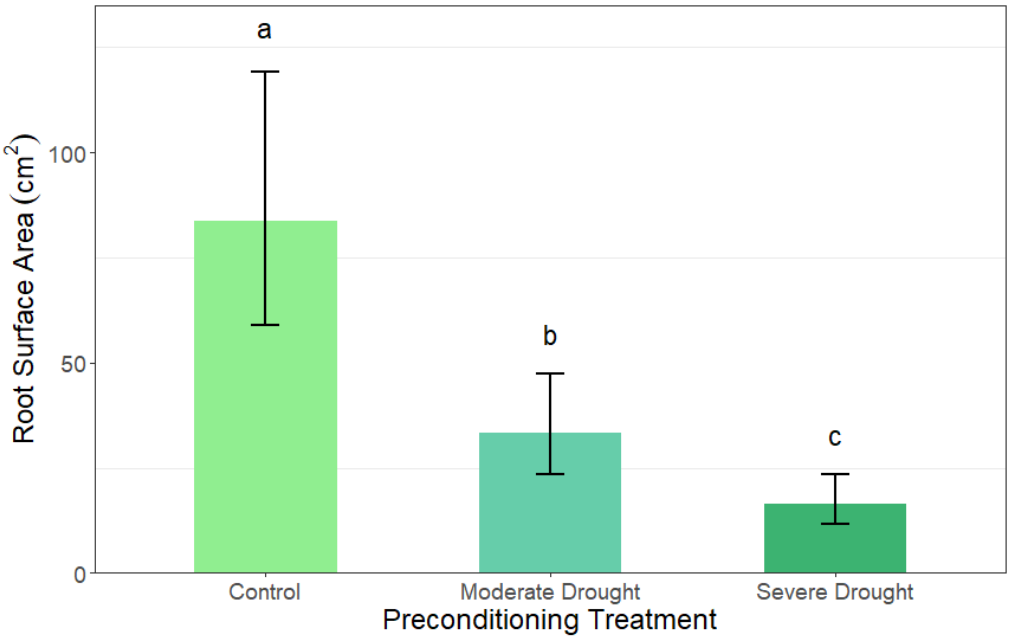

Figure 3.5. Mean back-transformed root surface area (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

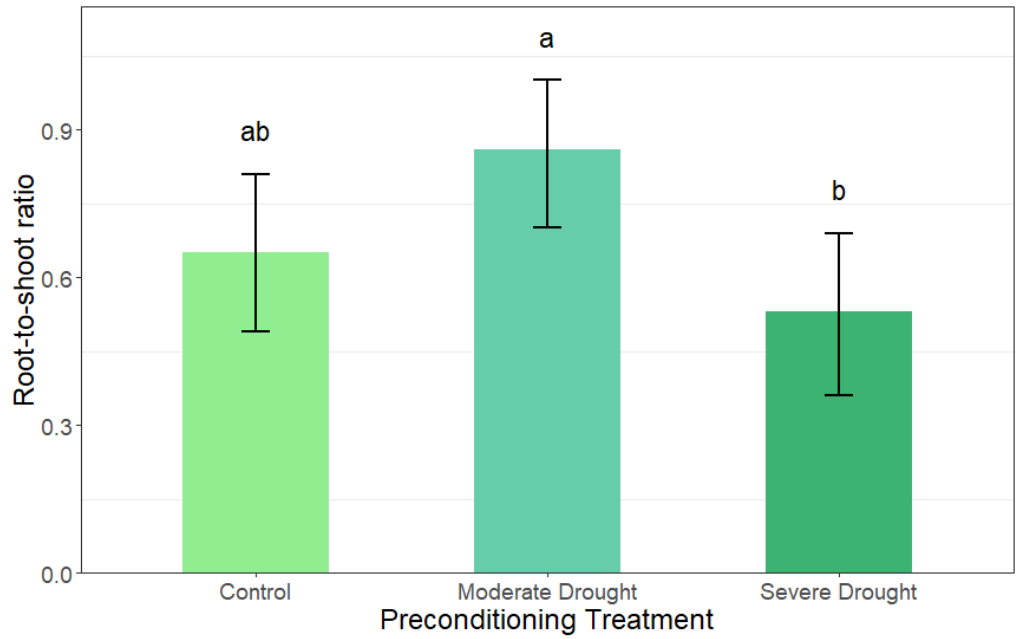

Figure 3.6. Mean root-to shoot ratio (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

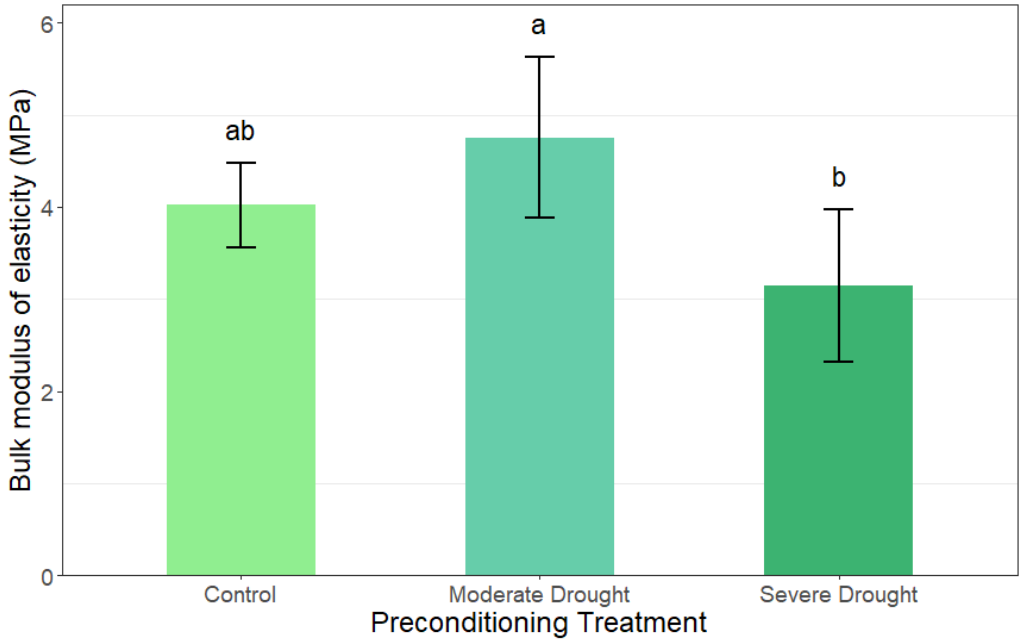

Figure 3.7. Mean bulk modulus of elasticity (ε) (with 95% confidence intervals) for drought preconditioning treatments. Different letters indicate significant differences ($p < 0.05$) among treatments.

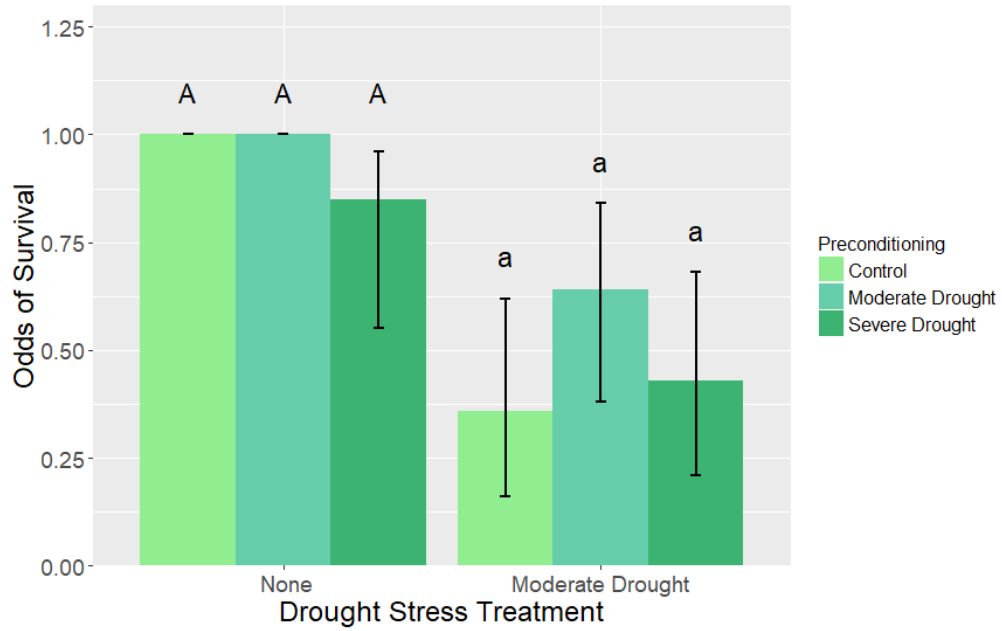

Figure 3.8. Mean odds of survival (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

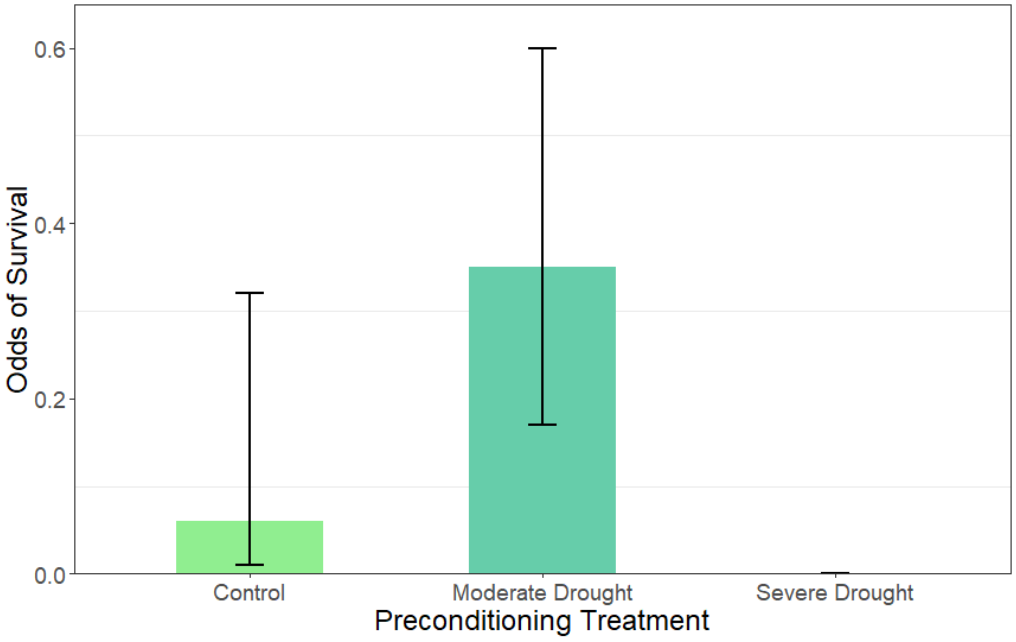

Figure 3.9. Mean odds of survival (with 95% confidence intervals) for drought preconditioning treatments in the severe drought stress treatment.

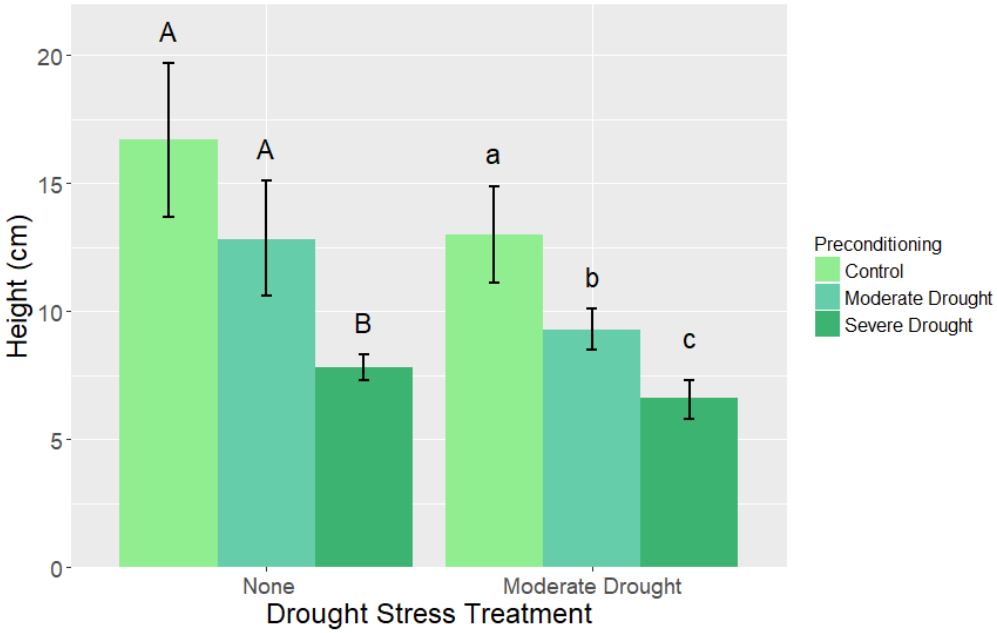

Figure 3.10. Mean height (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

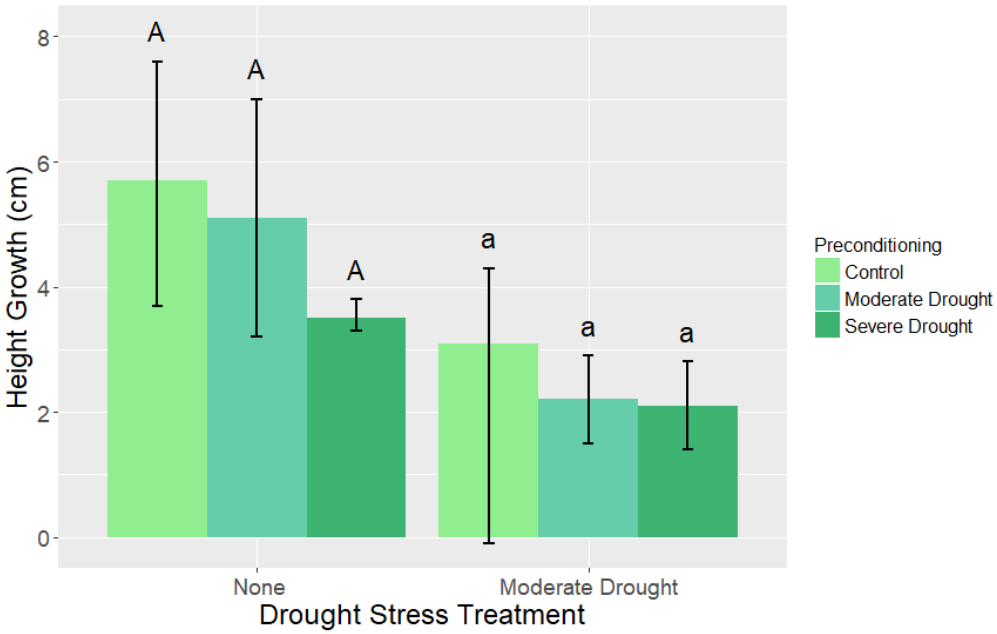

Figure 3.11. Mean height growth (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

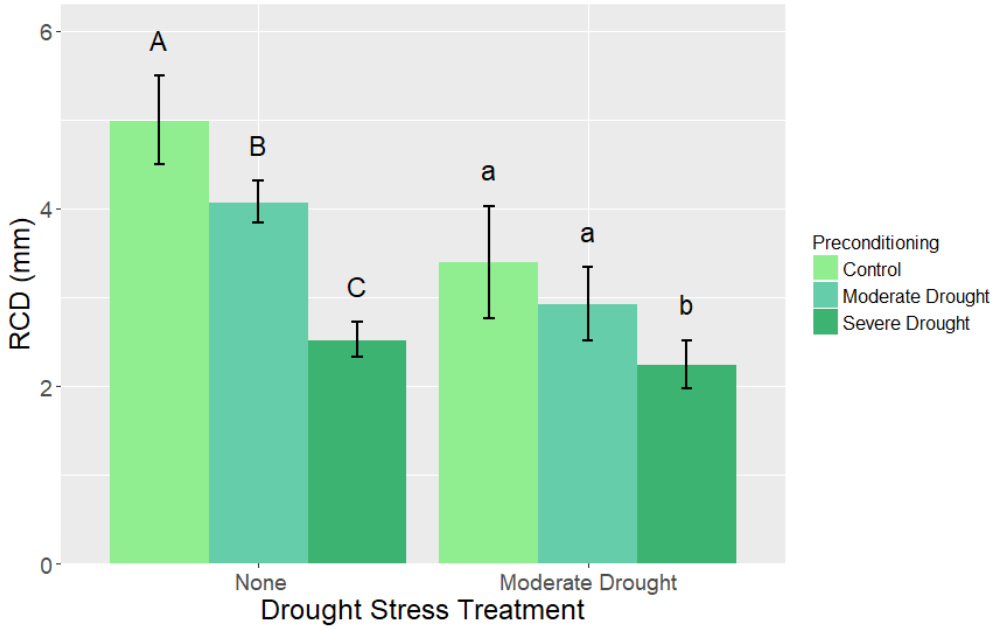

Figure 3.12. Mean root collar diameter (RCD) (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

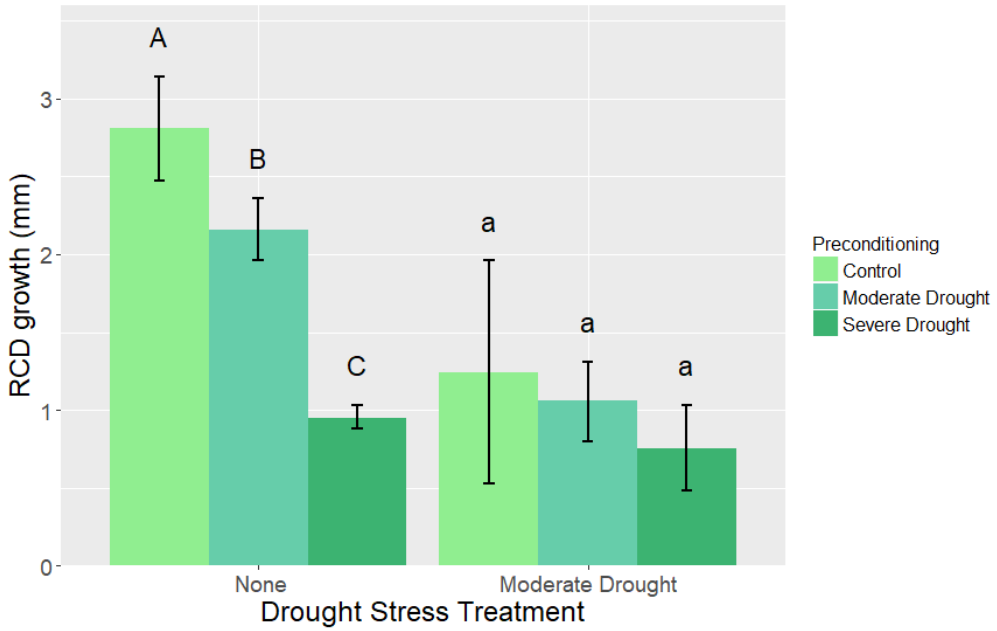

Figure 3.13. Mean root collar diameter (RCD) growth (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

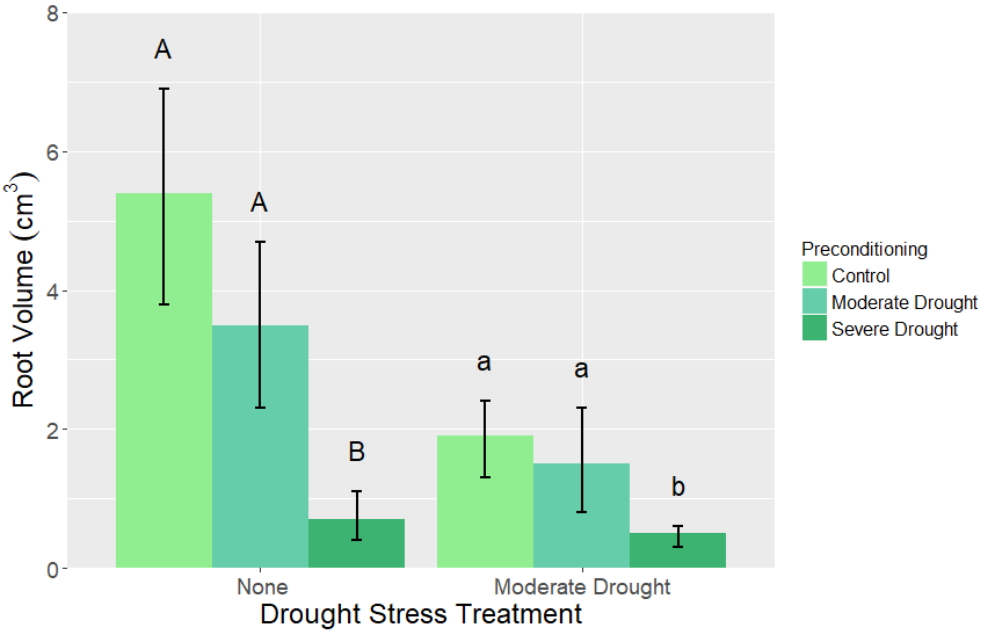

Figure 3.14. Mean root volume (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

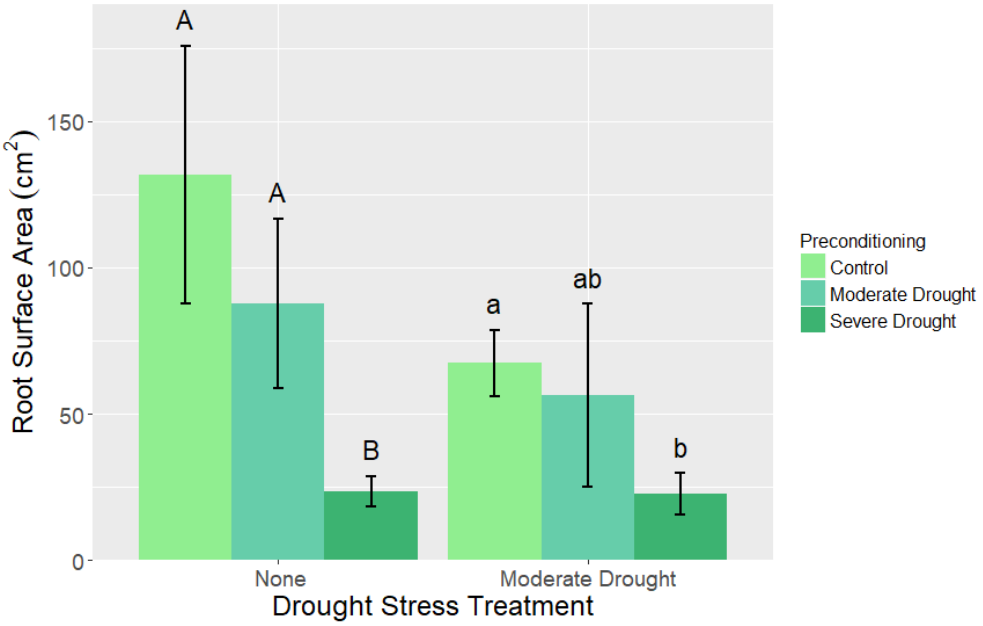

Figure 3.15. Mean root surface area (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

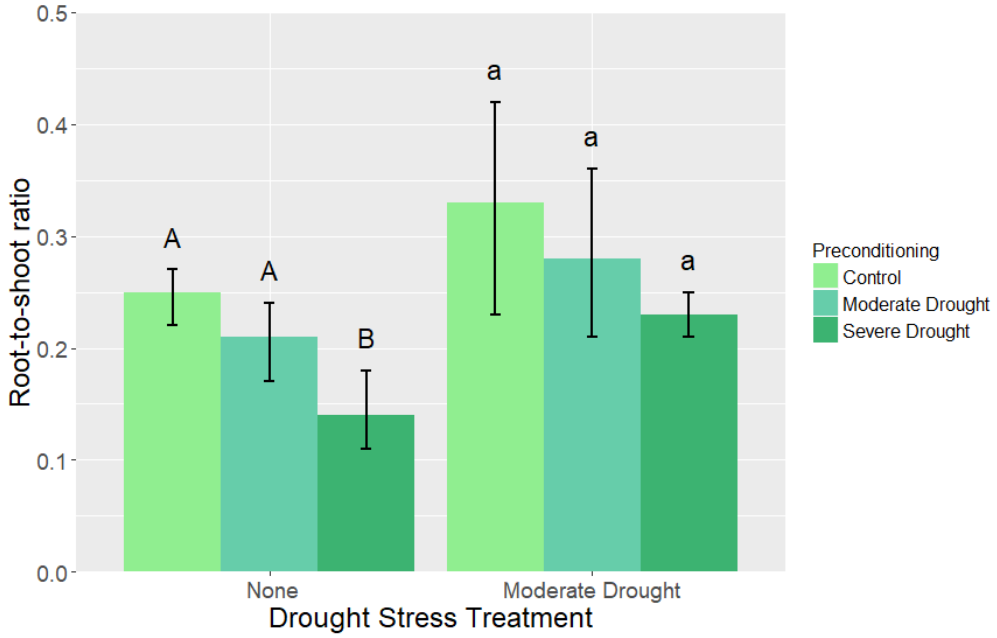

Figure 3.16. Mean root-to-shoot ratio (with 95% confidence intervals) for drought preconditioning treatments in the no drought and moderate drought stress treatments. Different letters within each drought level indicate significant differences ($p < 0.05$) among preconditioning treatments.

Chapter 4: Determining seed coat permeability and germination requirements of snowberry (*Symphoricarpos albus* (L.) Blake)

Abstract

Snowberry (*Symphoricarpos albus*) has been widely used in restoration because of its ability to tolerate disturbed sites and rapidly stabilize slopes. *Symphoricarpos albus* is most often propagated as cuttings, which reduces the genetic diversity in the resulting plantings. *Symphoricarpos albus* seeds have morphophysiological dormancy and may have an impermeable seed coat, which requires a prolonged period of warm and cold moist conditions (stratification) before germination can occur. These dormancy-breaking requirements are poorly defined, resulting in low germination rates in nurseries. The objectives of this study were to (1) determine whether the seed coat of *S. albus* is permeable to water, (2) the amount of time needed to reach full imbibition, and (3) the effect of moisture content on the length of warm and cold stratification needed to break dormancy. Results show *S. albus* has a permeable seed coat and reaches full imbibition between 26 and 27% moisture content (fresh weight basis). No *S. albus* seeds germinated after undergoing stratification treatments but seed viability remained high. The stratification treatments tested in this study did not break seed dormancy. It is unclear whether this is because warm or cold stratification temperature was inadequate (too high or constant as opposed to fluctuating to better reflect diurnal cycles), the stratification media was incorrect or if seeds required a subsequent regime of warm and cold stratification to allow germination.

Introduction

The environmental conditions required to alleviate seed dormancy for many native species are poorly defined or unknown. The type of dormancy dictates the treatments the seed must undergo before germination can occur. There are five dormancy types known in plants: physical, physiological, morphological, morphophysiological, and combinational dormancy. Physical dormancy occurs when the seed in unable to imbibe, or take up, water due to an impermeable seed coat or endocarp, which is the inner layer of the fruit (Baskin et al., 2000). Germination cannot occur until the barrier has been broken and the seed has imbibed water. Physiological dormancy occurs when the embryo is fully developed and can uptake water, but compounds within the seed or a physical structure in the seed prevents germination (Fenner and Thompson 2005). Oftentimes, the dormant embryo does not have enough force to grow through the endosperm or seed coat (Baskin and Baskin 2014). Morphological dormancy occurs when the embryo is not fully developed when the seed is dispersed and must undergo a growth period within the seed before germination can take place (Fenner and Thompson 2005). Morphophysiological dormancy (MPD) occurs when the seed has both morphological and physiological dormancy and combinational dormancy occurs when both physical and physiological dormancy is present (Baskin and Baskin 2014).

With the exception of physical dormancy, breaking all dormancy types requires that seeds be allowed to imbibe water (Baskin and Baskin 2014). Imbibition is controlled by the permeability of the seed coat or endocarp to water, the matric potential of the cell wall and internal tissues, and water availability in the surrounding environment (Woodstock 1988; Fenner and Thompson 2005). Imbibition is critical for protein synthesis, breakdown of storage compounds, embryo growth and modifying hormone levels within cells (Woodstock 1988; Hilhorst 2007; Debeaujon et al., 2007). The integrity and

structure of the seed coat is essential to preventing imbibitional injury (Baskin and Baskin 2014). Rapid imbibition can rupture seed membranes, causing cellular contents to leak and reducing seed vigor (Woodstock 1988).

Through research and trial and error, nurseries have developed multiple strategies to break dormancy in native seeds. Scarification is the process of damaging or destroying the seed coat or other seed layers to allow water to pass into the seed (Elias et al., 2012). Although scarification can be efficient at allowing water to pass into the seed, it can also damage seed tissues, causing death or abnormal seedling growth (Landis et al., 1998; Luna et al., 2014; Elias et al., 2012).

Stratification is the process where imbibed seeds are placed either under warm or cold temperatures and is widely used to break dormancy, especially in temperate species (Bonner et al., 1994; Kolotelo et al., 2001). The effect of each stratification type on seed physiology and morphology varies by species and even between individual seeds within a species (Milberg and Andersson 1998).

Warm stratification occurs between 18-30°C, with most temperate species requiring temperatures between 20-25°C (Landis et al., 1998; Baskin and Baskin 2014). The effects of warm stratification vary by species, but normally results in the breakdown of the seed coat or growth of underdeveloped embryos (Landis et al., 1998). Warm stratification can also be necessary to alleviate physiological dormancy (Baskin et al., 2002; Pipinis et al., 2012; Baskin and Baskin 2014). In *Prunus campanulata* and *Fraxinus excelsior*, warm stratification reduced levels of abscisic acid (ABA), a hormone crucial in the maintenance of dormancy (Chen et al., 2007; Finch-Savage and Leubner-Metzger 2006).

Cold stratification occurs at temperatures ranging from 1-10°C, with 5°C optimal for most temperate species (Baskin and Baskin 2014). In studies on *Arabidopsis,* cold temperatures activate a suite of genes, about a quarter of which regulate the synthesis of gibberellic acid (GA), a hormone

necessary for germination (Yamaguchi et al., 2007). Gibberellic acid concentration increases during cold stratification in many species with physiological dormancy (Chen et al., 2007; Finch-Savage and Leubner-Metzger 2006; Baskin and Baskin 2014). Cold stratification also increases the growth potential of embryos, allowing them to exert more force against the seed coat or other surrounding tissues and initiate germination (Carpita et al., 1983; Geneve 1991). The increase in embryo growth potential is possibly triggered by GA or by the accumulation of other compounds that decrease osmotic pressure in the embryo to facilitate water uptake and expansion (Chen et al., 2008; Geneve 1991). Embryo growth and the leaching of growth-inhibiting compounds also occur during cold stratification (Baskin and Baskin 2014).

In some species, the effectiveness of stratification at breaking dormancy is increased when alternating temperatures are used to mimic natural day and night temperature fluctuations (Baskin and Baskin 2014; Bewley and Black 1994). Seeds must remain fully hydrated during stratification. If seeds dry out, they could enter a secondary dormancy and germination will not proceed. Drying after imbibition could also lead to seed death (Baskin and Baskin 2014). In addition, seeds need to be well aerated during stratification to maintain metabolic reactions (Landis et al., 1998).

Previous studies on the genus *Symphoricarpos* suggest that the genus has morphophysiological dormancy and perhaps physical dormancy (Hidayati et al., 2001; Pelton 1953; Flemion 1934). The embryo is underdeveloped at dispersal and requires both warm and cold stratification to break dormancy. The initial studies on *Symphoricarpos* performed in the 1930s and 1940s suggest that the seed coat prevents imbibition and is degraded during warm stratification or by soaking in sulfuric acid to allow for water uptake (Flemion and Parker 1942; Flemion 1934). Those studies also suggest that the seed coat is an impediment to radicle emergence (Pfieffer 1934).

Only one recent study has been performed on any species of *Symphoricarpos* (Hidayati et al., 2001). This research concluded that the seed coat does not prevent water entry into the seed and does not mechanically restrain germination. This study also monitored embryo growth throughout warm and cold stratification and found that embryo growth occurred during cold stratification. This combination of traits suggests that *Symphoricarpos* has nondeep complex morphophysiological dormancy (Baskin and Baskin 2014). Honeysuckles (*Lonicera* spp.), a genus closely related to *Symphoricarpos*, also has morphophysiological dormancy, but stratification requirements to break dormancy vary between *Lonicera* species (Hidayati et al., 2000). There have been no studies investigating whether dormancy requirements vary between *Symphoricarpos* species.

There is no widely recognized standard protocol for propagating *S. albus*. In a propagation manual on growing Colorado native plants (Vories 1981), the author lists seven different propagation protocols for *S. albus*. Most protocols recommend a warm stratification for 3 to 4 months at temperatures between 22 and 30°C followed by cold stratification at 5°C for 4 to 6 months (Walker 2008; Rose et al., 1996; Wasser 1982; Vories 1981; Evans 1974; Flemion 1934). Multiple propagation manuals recommend soaking the seeds in sulfuric acid in place of warm stratification to soften the seed coat, though some manuals note that germination rates are reduced when compared to warm stratification (Walker 2008; Dirr 1998; Vories 1981; Evans 1974; Flemion 1934). A few manuals mention that sowing *S. albus* in flats and placing outside to warm and cold stratify under natural conditions leads to satisfactory germination (Wasser 1982; Vories 1981; Flemion 1934). Species in *Symphoricarpos* often germinate more readily the second year after dispersal, with only sporadic germination the first spring after dispersal (Hidayati et al., 2001; Pelton 1953; Flemion and Parker 1942). *Symphoricarpos albus* fruit often remain on branches throughout the late summer and fall, during which they are not imbibed and cannot undergo warm stratification until the summer after dispersal (Walker 2008; Baskin and Baskin

2014). Multiple propagation manuals mention that the seed coat is impermeable to water and warm stratification is necessary to soften the seed coat to allow for imbibition (Wasser 1982; Evans 1974; Pelton 1953; Flemion 1934).

Symphoricarpos albus is easy to propagate using cuttings and most nurseries use this method to grow this species (Walker 2008; Dirr 1998; Rose et al., 1998). Although it is a reliable and cost-effective method to produce plants, cuttings result in many plants that are genetically identical. A population composed entirely of cuttings will have less genetic variability than what would be found in a natural population or from a population grown from seed.

The importance of creating genetically diverse communities on restoration sites is becoming widely recognized. The level of genetic diversity within a population affects primary productivity, levels of biodiversity, rates of decomposition and nutrient cycling, susceptibility to disease and invasion by nonnative species and the ability to recover after a disturbance (Kettenring et al., 2014; Crutsinger et al., 2008; Hughes et al., 2008; Lesica and Allendorf 1999). Assembled communities with high genetic diversity within populations maintain greater species diversity over longer time periods (Booth and Grimes 2003). Ecosystems with higher genotypic diversity demonstrated a greater resilience and ability to recover from disturbance (Reusch et al., 2005). Developing effective and efficient methods of propagating *S. albus* from seeds, which will result in each plant being genetically distinct, will ensure that populations of *S. albus* on restoration sites will have the highest chance of successful establishment and growth.

The objective of this research is to determine if *S. albus* seed has physical dormancy and to compare germination rates of both imbibed and non-imbibed seeds among various stratification regimes. It was hypothesized that *S. albus* does not have physical dormancy and fully imbibed seeds will

require shorter stratification times and result in higher rates of germination than seeds placed into stratification while dry.

Methods

Snowberry seeds were collected in November 2016 near Sequim, WA (48.105241°N, 123.250944°W). Fruits were macerated and washed through a screen to remove pulp. The remaining pulp/seed mixture was dried, then the remaining chaff has removed from the seeds by rubbing through a screen. Seeds were stored at approximately 5°C with desiccant for 22 weeks. A tetrazolium test performed at the Oregon State University Seed Lab (Corvallis, OR, USA) in April 2017 found the seed lot had 94% viability. Two studies were completed and described below.

Imbibition Study

Seeds were randomly selected using seed industry protocol for hand sampling (AASCO 2006), placed in water and any floating seeds were discarded. Seeds were grouped into 29 samples with 50 seeds in each sample. Fifteen samples were sliced with a scalpel at the micropylar end of each seed, cutting completely through the endocarp. Fourteen samples were left intact. Each sample was placed in a mesh bag, weighed then placed in aerated distilled water. Samples were weighed every 4 hours for 12 hours, then every 12 hours until seeds had been soaked for a total of 60 hours. Samples were surfaced dried before weighing. Seven of the scarified samples and three of the non-scarified samples were not used for analysis because of seed loss during the experiment. The moisture content of dry seeds was determined by drying three samples of 150 seeds each into an oven set at 105°C for 24 hours (Elias et al., 2012). The average moisture content (on a fresh weight basis) was 6.37% and was used as the starting moisture content for all samples. The moisture content on a fresh weight basis was determined for each sample (Elias et al., 2012).

Imbibition and Stratification Study

Seeds were selected using the same hand sampling protocol as the imbibition study and placed into 126 groups of 60 seeds each. Seeds were cleaned in a hydrogen peroxide solution (3:1 water: hydrogen peroxide (3% concentration)) for 5 mins, then rinsed in running water for 10 mins and allowed to dry. Based on the results from the previous study, seeds were not scarified and half of the seeds were fully imbibed (placed in aerated water for 12 hours) and half were not imbibed.

Samples were randomly assigned one of 42 different stratification treatments (Table 4.1). Each stratification treatment had 3 replications. Warm moist stratification occurred in a single germination chamber (Hoffman Manufacturing, Corvallis, OR, USA) set at 25°C/15°C at 12 hour intervals. Cold moist stratification occurred in a fridge set at 5°C. While in stratification, seeds were placed in petri dishes containing 15 mL sterile sand and 5 mL distilled water and wrapped in aluminum foil. Distilled water was added to dishes when moisture content in the dish fell below 75% moisture content, determined by weight. The sand/seed mixture was aerated once a month by stirring. The location of the petri dishes in the germination chamber and fridge were randomized every week.

After a stratification treatment had been completed, seeds were placed in a germination chamber (Hoffman Manufacturing, Corvallis, OR, USA) set at 25°C/15°C at 12 hour intervals with 12 hours of light. Seeds were placed in 9cm petri dishes with 2 layers of Whatman No. 1 filter paper and 2.5mL of distilled water. Petri dishes were monitored daily for 30 days for germination and to maintain adequate moisture content. Seeds were considered germinated when the emerged radicle exceeded 1mm in length. Distilled water was added to a petri dish if the dish fell below 75% moisture content. Moldy seeds were removed. Due to low germination rates, seed viability was assessed via a tetrazolium test on the following treatments: (1) warm moist (WM) 60 days – cold moist (CM) 180 days, (2) WM 90

days – CM 150 days, (3) WM 90 days – CM 180 days, (4) WM 120 days – CM 120 days, (5) WM 120 days – CM 150 days, and (6) WM 120 days – CM 180 days.

Statistical Analysis

All analyses were done with R (Version 3.4.3, The R Foundation for Statistical Computing, 2017). For each experiment, all dishes were assumed to be independent. All effects of interest were estimated from the corresponding models. Differences between treatments for each parameter were analyzed using either one-way or three-way ANOVAs. The moisture contents for scarified and intact seeds were determined using a time series linear mixed model with a Gaussian correlation structure. A generalized linear model using a binomial distribution with the logit link was used to test the effect of seed moisture content, warm stratification length and cold stratification length on the odds of germination.

Results

Imbibition Study

There is evidence of an interaction between scarification and time ($p < 0.0009$) in determining the moisture content of *S. albus* seeds. There was no overall effect of scarification on seed moisture content ($p = 0.09$), though the differences between scarified and intact seeds were almost significant at the $\alpha = 0.05$ level (Table 4.2). Imbibition curves for scarified and intact *S. albus* seeds are found in Figure 4.1. Moisture content of snowberry seeds at full imbibition was between 26 and 27% (fresh weight basis).

Imbibition and Stratification Study

There was no germination in any of the imbibition and stratification treatments. The seeds retained high viability (> 90%) after undergoing stratification and it appears that dormancy-breaking requirements were not met during this study.

Discussion

Imbibition Study

Snowberry does not have a water-impermeable seed coat. Scarified seeds imbibe water at a quicker rate but within 12 hours the water contents of both scarified and intact seeds were similar and remained stable between 26 and 27% moisture content (fresh weight basis) (Figure 4.1). These results match previous research that found that *Symphoricarpos orbiculatus* does not have physical dormancy (Hidayati et al., 2001).

Imbibition and Stratification Study

The stratification treatments did not alleviate seed dormancy. There was no germination during the experiment but, based on the tetrazolium tests performed on the last six treatments to be placed in the germination chamber, there was no loss of seed viability. Thus, the conditions required to break dormancy were not met.

The experimental design was based on protocols from native plant manuals (Walker 2008; Rose et al., 1996; Wasser 1982; Vories 1981; Evans 1974). Warm stratification took place at the recommended temperature (25°C/15°C), which was also the warm temperature combination used by Hidayati et al. (2001) to germinate coralberry (*S. orbiculatus)*. The average refrigerator temperature during cold stratification in this experiment was 5.5°C (± 1.5°C). Propagation handbooks recommend snowberry be cold stratified at 5°C (Walker 2008; Rose et al., 1996; Wasser 1982; Vories 1981; Evans 1974), with the optimal temperature range for cold stratifying temperate species between 1 and 5°C (Landis et al., 1998). Growth in the underdeveloped embryo in *Symphoricarpos* occurs during cold stratification (Hidayati et al., 2001) and it is possible that the temperature was not cold enough to allow for adequate growth in the embryo in this experiment.

In a study on *S. orbiculatus*, researchers were able to germinate seeds after exposure to a summer – fall – winter – spring temperature sequence (25°C/15°C→ 20°C/10°C → 15°C/6°C → 5°C → 15°C/6°C) (Hidayati et al., 2001). The summer, fall and spring regimes contained alternating temperatures to simulate differences in day and night temperatures. The same study found no germination when *S. orbiculatus* was exposed to 12 weeks at 25°C/15°C followed by 12 weeks at 5°C. There is research that suggests some species have higher germination rates when exposed to fluctuating rather than constant temperatures during cold stratification (Baskin and Baskin 1998; Milberg and Andersson 1998; Bewley and Black 1994; Dillon and Forcella 1985). Alternating temperatures more closely mimic conditions that seeds would be exposed in natural environments (Baskin and Baskin 2014). Some nurseries are able to germinate *S. albus* by placing sown seeds outdoors during the fall, winter and spring (pers. observ.). Different populations of a species can have different dormancy requirements (Milberg and Andersson 1998) and even though propagation manuals recommend *S. albus* be cold stratified at 5°C, there may be some populations that require alternating temperatures to alleviate dormancy. *Symphoricarpos albus* is also known to have sporadic germination that can occur over multiple years (Walker 2008; Hidayati et al., 2001; Flemion and Parker 1942).

Other factors such as gas exchange and water availability can affect whether dormancy conditions are met (Baskin and Baskin 2014; Landis et al., 1998). Seeds were kept at the ideal moisture level and were aerated during the experiment and maintained high viability throughout the experiment, so it is less likely that these factors could have played a role in preventing the breaking of dormancy. The temperature at which the seeds were germinated may have also affected the germination rate. The seeds were kept at the standard germination temperature recommended for temperate seeds (25°C/15°C at 12 hour alternating intervals) (Baskin and Baskin 2014), but germination may have been enhanced under cooler temperatures that replicate spring temperatures more closely (Hidayati et al.,

2001; Flemion and Parker 1942). The seeds were placed in sterilized sand during stratification. One study states that peat moss is a better medium for stratification because its slight acidity helps to break down the seed coat (Pfieffer 1934), but only one current propagation manual states that peat moss can be used for stratification (Vories 1981). Most current propagation manuals do not state that peat moss is a requirement for stratification (Walker 2008; Dirr 1998; Rose et al., 1998) and one manual recommends using sand (Wasser 1982).

Conclusion

Future research should perform stratification experiments with a mixture of alternating and constant temperatures, different stratification media and various germination temperatures. Based on how quickly *S. albus* seeds imbibe water, the initial water content of the seeds when placed into stratification may be irrelevant to how quickly dormancy can be broken.

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Table 4.1. Stratification treatments for *Symphoricarpos albus*. Fully imbibed seeds were soaked in aerated distilled water for 12 hours. Both stratification treatments occurred in the dark. Warm stratification occurred at 25°C/15°C alternating every 12 hours and cold stratification occurred at 5.5°C (±1.5°C).

Imbibition Level	**Warm Stratification (days)**	**Cold Stratification (days)**	**Imbibition**	**Warm Stratification (days)**	**Cold Stratification (days)**
Control (Dry)	0	0	Fully Imbibed	0	0
		90			90
		120			120
		150			150
		180			180
	30	90		30	90
		120			120
		150			150
		180			180
	60	90		60	90
		120			120
		150			150
		180			180
	90	90		90	90
		120			120
		150			150
		180			180
	120	90		120	90
		120			120
		150			150
		180			180

Table 4.2. Results of the analysis for the effects of scarification, time and their interaction on seed moisture content of *Symphoricarpos albus.*

Source of Variation	Time *P*	Scarification *P*	Time x Scarification *P*
Moisture content	< 0.0001	0.0870	0.0009

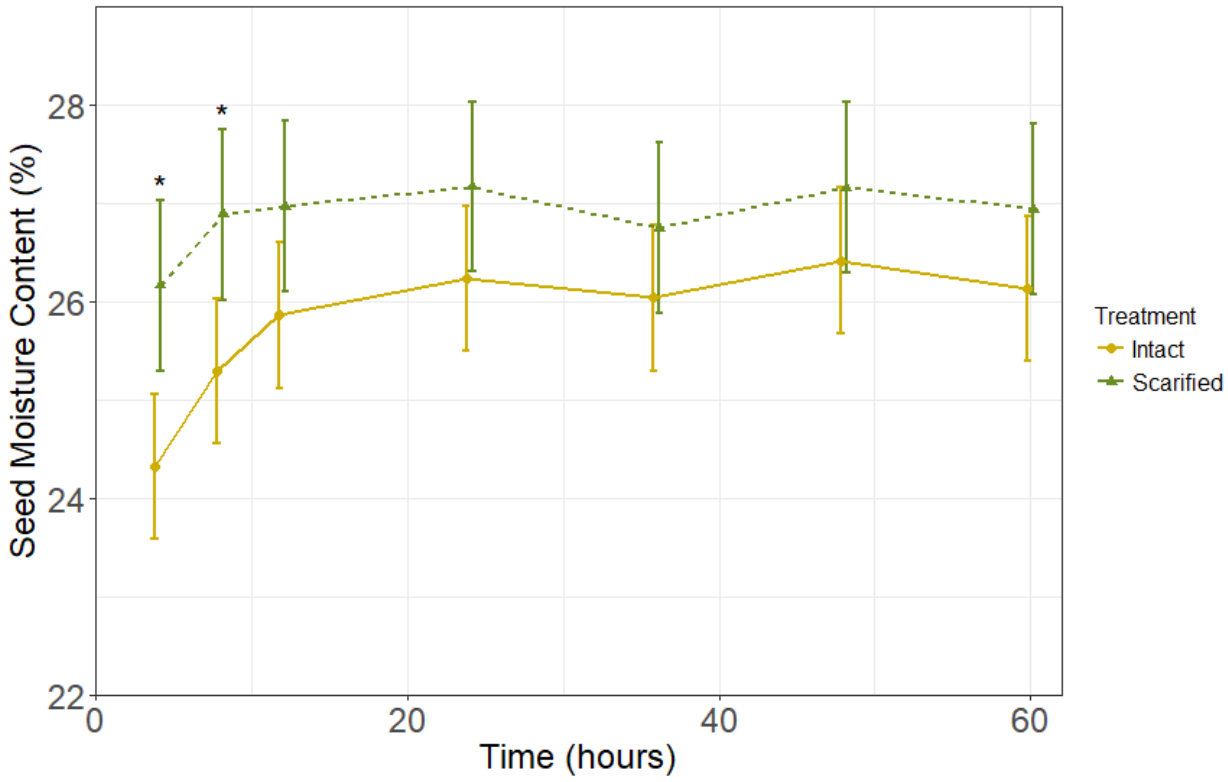

Figure 4.1. Water uptake curve (fresh weight basis) for *Symphoricarpos albus*. All seeds were assumed to have a dry moisture content of 6.37%. An asterisk (*) represents a significant difference ($p < 0.05$) in mean moisture content between scarified and intact seeds.

Chapter 5: General Conclusion

Producing quality seedlings is an integral step in a successful restoration or afforestation project. Despite the common misperception that growing plants is simple, native plants have a myriad of requirements for germination and healthy growth, many of which are not known. Developing propagation protocols that are efficient and cost-effective is crucial for producing seedlings that have the highest chance of survival and growth on the site where they will be planted.

The germination protocols for *Pinus occidentalis* developed from this research require only low-cost resources that are locally available to Hispaniolan nurseries and, when compared to current protocol, reduces the risk of fungal contamination that could weaken or kill seedlings. Drought is one of the most impactful environmental stresses that seedlings experience after planting and developing a methodology to tailor a plant to the water availability of a site will increase seedling survival. This study provides a framework to produce *P. occidentalis* seedlings with the optimal morphology and physiology to survive on sites that experience a wide range of water stress.

Although the study on *Symphoricarpos albus* did not clarify the stratification requirements needed to break dormancy, it can guide future research by expanding the different stratification treatments that should be researched, such as increasing the range of temperatures studied and the effect of alternating versus constant temperatures on breaking dormancy. Determining that the seed coat is permeable to water will prevent nursery managers from unnecessarily scarifying seeds and potentially damaging the embryos. Nonetheless, more research is needed to make growing *S. albus* from seed economically and technologically feasible.

The scope of inference for the results of these studies is limited. Seeds from only one population each of *P. occidentalis* and *S. albus* were tested. The degree of dormancy can vary among years,

different populations of a single species and even among seeds from the same plant (Fenner and Thompson 2005; Baskin and Baskin 2014). Downie et al. (1998) found that the germination rate of different white spruce (*Picea glauca*) populations responded differently to the moisture content of the seed during stratification and germination. Donald (1981) recorded that different *Pinus patula* populations responded differently to the same cold stratification and soaking treatment, with the germination rate of two populations increasing, the germination rate of two populations decreasing and seven populations showing no change in germination rate. A study of annual plants in southern Sweden found that 70% of species showed differences in germination rates among populations, even after stratification (Milberg and Andersson 1998). The elevation of the parent plant can also affect the stratification requirements of seeds (Dorne 1981; Weber and Sorensen 1992). The results of this research should be used as a basis for future study into dormancy and germination pretreatment requirements for *P. occidentalis* and *S. albus*. Future research should utilize seed sources from different locations and different years that vary in precipitation, elevation and temperature extremes.

There can also be variation in how different populations respond to drought stress, with some species showing high levels of phenotypic plasticity, with seedlings from different populations showing a similar response to drought (Heschel et al., 2004). In other species, separate populations evolve local adaptations in response to drought (Ramirez-Valiente et al., 2010; Ma et al., 2014). In maritime pine (*P. pinaster*), changes in water relations parameters in response to drought differed based on seed provenance (Fernandez et al., 1999). In contrast, different families of loblolly pine (*P. taeda*) had no difference in water stress response (Seiler and Johnson 1988) or survival and growth after 13 years (South et al., 1985). In black spruce (*Picea mariana*), more genetic variability occurs within a population than between populations, with no difference in response to drought preconditioning between black

spruce from different elevations (Zine El Abidine et al., 1994). Not enough research has been done on *P. occidentalis* to elucidate phenotypic plasticity of the species or ecotypic differences among populations.

The practices suggested in these studies are easy to implement and are economically feasible for small nurseries. Improving propagation and production practices will help nurseries meet the growing demand for high quality seedlings and improve the survival and long-term success of seedlings on restoration and afforestation sites.

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