A Comparison of Drought Tolerance in Two Conifers with Contrasting Mycorrhizal Associations

By Bethany Robson

Supervised by Dr. Barbara Hawkins

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Supervisory Committee:

Dr. Barbara Hawkins, Supervisor, Department of Biology

Dr. Steve Perlman, Honours Advisor, Department of Biology

Dr. Patrick Von Aderkas, Examiner, Department of Biology

Abstract

Drought events are increasing in frequency, severity, and distribution as a result of climate change. Plants have a variety of adaptations to water stress, including symbioses with mycorrhizal fungi. Little is known about how the type of mycorrhizae (arbuscular or ecto-) may affect drought tolerance, especially in conifers that are restricted in what association they can make. Research suggests that there may be an effect of mycorrhizal type on drought tolerance, and that mechanisms for this may be different in arbuscular and ecto- mycorrhizae. The objective of this study was to determine how the type of mycorrhizae may affect drought tolerance in *Pinus contorta* and *Thuja plicata*, species that make contrasting mycorrhizal associations. Three experiments were performed using both aeroponics and traditional soil culture to explore the effects of mycorrhizal association on drought tolerance. *P. contorta* performed consistently better in all experiments when compared to two populations of *T. plicata* from different ecozones in British Columbia. Quantum yield declined linearly with increasing drought stress in both treatments with mycorrhizal colonization, and non-linearly in the treatment with no colonization. These trends were consistently shown across all seedling populations, which suggests that both types of mycorrhizal symbioses are important in the drought tolerance of these species. Further investigation is needed to determine how mycorrhizae may influence recovery after drought in these species, as well as the mechanisms mycorrhizae may use to improve drought tolerance in host plants like *P. contorta* and *T. plicata*.

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1. Introduction

1.1 Drought in Forest Ecosystems

Climate change is affecting the frequency, severity, and distribution of drought events across the globe. Drought events are of concern globally, but forest ecosystems are at an increased risk (Batllori *et al*, 2020). Forests that are in historically wet climates are less likely to adapt to the expected changes than those already in areas with dry conditions. (Helman *et al*, 2017). This leaves wetter forests, like the temperate rainforests found in British Columbia, particularly vulnerable.

Drought is a complex disturbance force, and the effects are disproportionate. While dry conditions can directly cause mortality in trees, these conditions are often coupled with other stressors like heat (Gazol and Camarero, 2022). Seedlings are especially at risk of mortality, and mature trees that survive have been shown to be more vulnerable to pathogens or pests (Hossain *et al*, 2019; Han *et al*, 2022). The effects of a drought event vary spatially across range distributions of species and are particularly impactful at the drier edges of a species' range (Anderegg *et al*, 2019). Drought can have a significant effect even in cases where it is not severe or in areas that already experience regular dry periods. Therefore, changes to these events can have significant consequences. Repeated drought in boreal forests has resulted in greater mortality even with mild sequential drought events, with the highest mortality rates occurring in the areas that traditionally receive the most precipitation (Sánchez-Pinillos *et al*, 2022). Drought induced by regular periodic events such as El Nino has been shown to have a significant effect on seedling mortality in seasonally dry tropical forests, especially in evergreen species (Nutiprapun *et al*, 2023).

The severity of drought effects on trees is dependent on a variety of factors. Tree density is negatively correlated with drought tolerance and tree resilience, indicating that forests with species capable of growing at greater density, such as *Pinus contorta*, are at greater risk in drought events (Bottero *et al*, 2017). Tree height and leaf traits were shown to be significant factors in drought tolerance in a temperate broadleaf forest, suggesting that taller trees may be more susceptible to drought, and species capable of slowing turgor loss in leaves have an advantage in drought events (McGregor *et al*, 2021). Many of these factors are based on individual tree characteristics. Stand level drought effects are also present. Competition between trees and overall species composition are both factors in forest resilience, and forests with a greater diversification of traits amongst species may be more capable of withstanding a drought event (Pretzsch *et al*, 2022).

1.2 Water Stress and Drought Tolerance in Plants

Water stress in plants can be compromising for both short-term functionality and long-term growth. Furthermore, effects of water stress can be exacerbated when disturbance forces are combined, such as when dry conditions occur along with high temperatures (Xu *et al*, 2020). Insufficient amounts of water can lead to morphological, physiological, and biochemical changes. Water stress slows the rate of photosynthesis, reducing carbon uptake and carbohydrate synthesis (Seleiman *et al*, 2021). It also results in the production of reactive oxygen species (ROS) that cause oxidative damage to tissues. Prolonged water stress can result in changes to membrane integrity or permeability and protein denaturation. Severe water stress can also result in cavitation, which has been shown to be a primary driver of plant mortality and tissue loss

(Lens *et al*, 2022). Combined, these effects have the potential to impact the long-term health and success of the plant.

Plants have a variety of mechanisms to protect against the effects of drought, if they are unable to escape or avoid it. Plants have several dynamic responses that occur in water stress (Seleiman *et al*, 2021). Stomatal closure is one such response, causing evapotranspiration and CO_2 assimilation to slow or stop. Plants can perform osmotic adjustment to maintain water potentials in various tissues. In addition, plants generate a variety of antioxidant compounds to counteract the accumulation of ROS. In prolonged water stress, root growth is altered to increase the surface area available for water uptake. Plants use these and other mechanisms in different ways to maximize their functionality. Plants can also be broadly categorized as isohydric or anisohydric, based on water stress mitigation strategies (Jin *et al*, 2023). Isohydric plants prioritize maintenance of water potential at the cost of stomatal conductance, while anisohydric plants prioritize stomatal conductance at the cost of fluctuating water potential. These strategies have some correlation with general drought tolerance, as xeric species may be more likely to adopt isohydric strategies while mesic species may adopt anisohydric strategies.

1.3 Mycorrhizae and Drought Tolerance

A factor in drought tolerance may be the formation of symbioses with mycorrhizae. Mycorrhizae are symbiotic fungi that associate with plant roots (Smith and Read, 2008). They use their network of hyphae within the soil to bring water and nutrients to the host plant and receive carbohydrates from the plant in return. Several types of mycorrhizae exist, but the majority belong to two main groups: arbuscular mycorrhizae (AM) and ectomycorrhizae (ECM). AM are members of Glomeromycota, which are represented by fewer than 200 species. In

contrast, ECM are primarily members of Basidiomycota and Ascomycota and are represented by almost 8000 species. Symbioses with AM are widespread among over 70% of vascular plants, while ECM symbioses tend to be more host-specific and are formed with less than 3% of seed plants.

AM and ECM can be distinguished by growth habit and general structure, where they vary in cell penetration and hyphal constructs (Figure 1). AM grow within the cell walls of the root in the host plant and create distinctive hyphal structures within the root, known as arbuscules. Some species of AM are strictly vesicular, making no arbuscules at all (Smith and

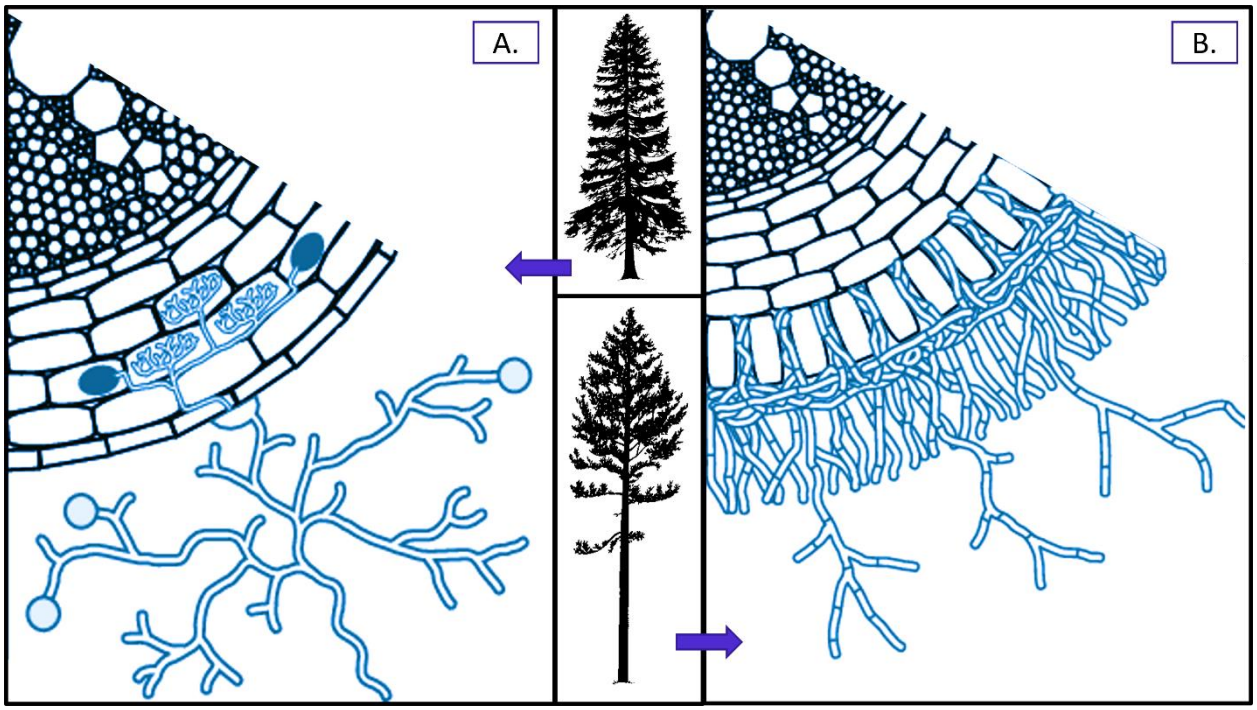

Figure 1. Diagram of two root cross sections with contrasting mycorrhizal types, modified from Péret (2009). Fungal tissue is shown in light blue. **A.** A root cross section containing arbuscular mycorrhizae. *Thuja plicata* only make associations with these mycorrhizal fungi, as indicated by the arrow. **B.** A root cross section containing ectomycorrhizae. *Pinus contorta* only make associations with these mycorrhizal fungi, as indicated by the arrow. *Silhouettes used with permission from Natural Resources Canada, Canadian Forest Service.*

Reed, 2008). In contrast, ECM restrict their growth to the apoplast of the root and construct a

hyphal network in the root cortex (the Hartig net), as well as a hyphal mantle that surrounds the root tip. Both types of mycorrhizae have been shown to assist in uptake of various nutrients in addition to water, including phosphorus and nitrogen.

Mycorrhizae have been investigated for their role in improving the drought tolerance of their host plants. Much of the current research has been directed toward agricultural plants like maize, soybean and tomato. In these plants, arbuscular mycorrhizae have been shown to improve drought tolerance through the enhancement of a wide array of plant processes (Abdalla and Ahmed, 2021; Hu *et al*, 2020). Some studies have also focused on AM in trees. AM association improved seedling resilience in *Handroanthus serratifolius* (Correira *et al*, 2022), and in *Casuarina equiestifolia,* the association with AM led to improved growth and heightened peroxidase activity, which improved seedling drought tolerance (Zhang *et al*, 2010). This effect was also mirrored in a study completed on *Olea europaea,* showing that jasmonates and abscisic acid were reduced in *O. europaea* leaves, while carbohydrates, hormones such as auxins and salicylic acid, and other solutes were increased, resulting in an increase to drought tolerance (Takeya *et al*, 2022). Another study by Tao and colleagues (2022) showed that AM enhanced drought tolerance by altering MAPK genes, downregulating MAPK genes that are associated with drought sensitivity while increasing others associated with drought tolerance. Together, these studies indicate that AM can have a positive influence on drought tolerance in many plants.

Very little is known about how ECM may enhance drought tolerance, as many of the current studies focus on plants that are unable to associate with ECM. In contrast to AM, ECM form a relatively thick hyphal mantle surrounding the root tips, which could potentially act as a protective barrier between the root and dry soil. Some studies suggest that ECM may influence

nutrient dynamics during drought, but many of the mechanisms remain unclear (Liese *et al*, 2018; Li *et al*, 2021) The few studies investigating ECM and drought tolerance have primarily investigated species that make associations with both types of mycorrhizae. Both *Alnus* and *Populus* species can make associations with AM and ECM, and in these trees, AM have been shown to improve drought tolerance more than ECM (Kilpeläinen *et al*, 2020a; Kilpeläinen *et al*, 2020b). However, this research excludes trees that only associate with ECM. Many tree species associating with ECM are integral components in forest systems found in northern latitudes, so improving knowledge on this topic is of vital importance.

1.4 Study Overview

My research aims to investigate the role of mycorrhizae in the drought tolerance of two conifer species that associate with contrasting mycorrhizal types. A study completed on temperate forest deciduous trees with contrasting mycorrhizal types has suggested that there are differences in water stress responses between trees that associate with AM versus ECM (Liese *et al*, 2018). To investigate how mycorrhizal type may affect conifer drought tolerance, two tree species, *Pinus contorta* and *Thuja plicata*, will be subject to drought under both mycorrhizal and non-mycorrhizal conditions. Two populations of *T. plicata* from different biogeoclimatic zones in British Columbia will be used to determine if native precipitation regime has any effect drought tolerance with or without mycorrhizal colonization.

1.4.1 Study Species

P. contorta is a tree species native to North America. It is widely distributed, ranging from SE Alaska to California, USA and across much of the Western half of the continent as

various subspecies (Spellenberg *et al*, 2014). It creates a dense canopy and is a dominant tree species in the interior of British Columbia. It is tolerant of dry conditions and proliferates in early succession of disturbed forests, using fire as part of its reproductive strategy. In addition to its ecological importance, it is also a valuable commercial species (Jang *et al*, 2019). *P. contorta* associates with ectomycorrhizae and utilizes an isohydric strategy for minimizing drought stress by prioritizing the maintenance of water potential at the cost of stomatal conductance (Bradbury *et al*, 1998; Guo *et al*, 2022).

Thuja plicata is a tree species native to Western North America, and is found along the West Coast of North America, with some interior populations found in areas where conditions are right (Spellenberg *et al*, 2014). It is generally associated with *Pseudotsuga menziesii* and *Tsuga heterophylla*. *T. plicata* is tolerant of nutrient poor or swampy soils and is generally considered stress tolerant, with the exception of drought stress (Antos *et al*, 2016). It can proliferate in both disturbed and established forests. This tree species is extremely important in Indigenous cultures in the Pacific Northwest and is a valuable commercial species. *T. plicata* associates with arbuscular mycorrhizae and utilizes an isohydric strategy for minimizing drought stress (Warren *et al*, 2003; Weber *et al*, 2005; Gorzelak *et al*, 2017). In this study, one population is from the west coast in moist maritime conditions and the other is from the interior of British Columbia where conditions are inherently drier.

1.4.2 Rational for Treatments

In this study, three treatments will be used to elucidate a better understanding of how different mycorrhizal types affect drought tolerance in these two conifer species. The first treatment will occur in aeroponics with the roots treated to prevent mycorrhizal growth. This

treatment will provide a baseline drought tolerance for the species when no mycorrhizal symbioses are present in aeroponics. This treatment will be hereafter referred to as the non-mycorrhizal-aeroponic treatment. The second treatment will occur in aeroponics after the roots have been grown in inoculated soil to encourage mycorrhizal growth. Doing so will allow exploration of how mycorrhizal colonization may affect drought tolerance without the influence of soil. This treatment will be hereafter referred to as the mycorrhizal-aeroponic treatment. Finally, the third treatment will occur in inoculated soil to simulate natural conditions. This treatment will be hereafter referred to as the mycorrhizal-soil treatment.

Several measures have been selected to assess drought stress in these treatments. Chlorophyll fluorescence was chosen to non-destructively assess quantum yield (Y(II)) as a proxy for photosystem health (Maxwell and Johnson, 2000). During photosynthesis, light energy can be used in photochemistry, dissipated as heat, or released as fluorescent radiation. Because these three outcomes are linked, it is a useful determinant of changes to photosystem II. As drought stress can cause oxidative damage to photosystems, this will help to determine how drought stressed the seedlings are. Y(II) will be used instead of Fv/Fm because the seedlings will be assessed when non-photochemical quenching pathways have been activated, which is a more sensitive indicator of drought stress. Stomatal closure is a mechanism regularly utilized by plants to alleviate drought stress, as mentioned above. Therefore, gas exchange will be measured to determine the amount of stomatal closure occurring as the seedlings are subjected to extreme drought. Because high tension in the water column can cause cavitation, resulting in tissue loss and mortality, stem water potential will also be assessed.

1.5 Main Objectives

The objective of this study is to determine whether association with mycorrhizae improves drought tolerance in *Pinus contorta* and *Thuja plicata*, and if there is any difference in relative response to drought between *Pinus contorta* associated with ECM versus *T. plicata* associated with AM. I predict that in the non-mycorrhizal-aeroponic treatment, *Pinus contorta* will tolerate drought stress better than *Thuja plicata*, which will be indicated by less negative water potentials, higher rates of gas exchange, and higher Y(II) values. In the mycorrhizal-aeroponic treatment, I predict that *Pinus contorta* will tolerate water stress better than *Thuja plicata*, but that the difference in measured parameters will be more pronounced than in the non-mycorrhizal-aeroponic treatment. I predict that this trend will mirror that of the mycorrhizal-soil, which is representative of natural conditions. Finally, I predict that coastal *T. plicata* seedlings will have more negative water potentials, lower rates of gas exchange and lower Y(II) values than interior *T. plicata* seedlings that experience dry conditions more regularly.

2. Methods and Materials

2.1 Experimental Overview

Thirty soil-grown seedlings each of *P. contorta*, interior *T. plicata*, and coastal *T. plicata* populations were obtained before the study period for three treatments. *P. contorta* seedlings were obtained from Mr. S. Kiiskila of Arbutus Grove Nursery. Both populations of *T. plicata* seedlings were obtained from Dr. C. Filipescu of the Pacific Forestry Centre. The *P. contorta* seedlot originated from a Coastal Western Hemlock (CWH) western very dry maritime zone (49.23^{o} N, 124.67^{o} W) with an elevation of 500m. The interior *T. plicata* seedlot originated in a CWH dry maritime zone on the mainland of BC (49.68 ^{o} N, 122.57^{o} W) with an elevation of

1200m. The coastal *T. plicata* seedlot originated in a CWH submontane very wet maritime zone on Vancouver Island (50.54° N, 126.88° W) with an elevation of 166m. All experiments were conducted in a CMP3023 growth chamber (Controlled Environments Ltd., Winnipeg, Canada) using a 16:8hr day/night cycle at 20°C under a 3Red:1Blue LED light array producing 100-150 µmol photons m^{-2} s^{-1}.

The thirty seedlings of each population were divided among three treatments: non-mycorrhizal-aeroponic, mycorrhizal-aeroponic, and mycorrhizal-soil (Figure 2). Seedlings in the non-mycorrhizal-aeroponic treatment were grown for the entire experiment in aeroponic culture.

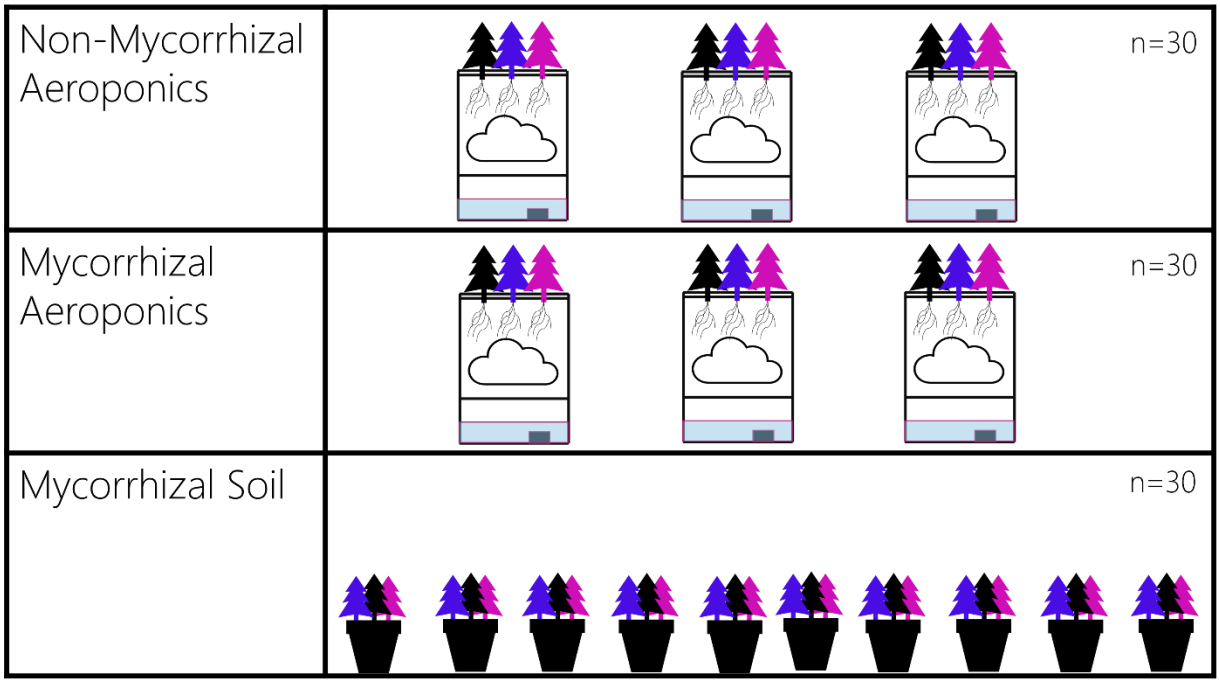

Figure 2. Summary of treatments in sequence. Mycorrhizal treatments were inoculated to encourage growth of mycorrhizae and were grown in soil while the non-mycorrhizal aeroponics treatment was underway. Colours represent the three seedling populations.

Seedlings in the mycorrhizal-aeroponic treatment were grown first in soil, followed by aeroponic

culture. Aeroponic culture was conducted in a nested bucket system assembled according to a custom design, see Figure S1 (Sarah Lane, personal communication). Three buckets, each holding 10 seedlings were used for aeroponic culture.

2.2 Aeroponic Growth

Plants in the aeroponics systems were grown in the following manner. Ten seedlings from each population were transferred to aeroponics for a total of thirty seedlings per treatment. Three to four seedlings from each population were allowed to co-culture in each bucket for a total of ten seedlings per system. 3L of nutrient solution was added to each bucket and replaced weekly. The buckets were monitored between solution replacements and topped up with 250ml of solution as needed to maintain system function. All nutrient solution was mixed using a modified ½ strength Hoagland's solution containing 6mM KNO_3, 4mM $Ca(NO_3)_2 \cdot 4H_2O$, 1mM HEPES Free Acid, 1mM $NH_4 \cdot H_2PO_4$, 2mM $MgSO_4 \cdot 7H_2O$, 50µM EDTA(Fe(III)), 46µM H_3BO_3, 9µM $MnCl_2 \cdot 4H_2O$, 0.8µM $ZnSO_4 \cdot 7H_2O$, and 0.3µM $CuSO_4 \cdot 5H_2O$ per litre.

2.3 Soil Growth

Plants grown in soil were treated in the following manner. At the beginning of the study period, twenty seedlings from each population, totalling sixty seedlings, were transferred into 4 L pots containing a 3:1 mix of Sunshine® #4 potting mix (Sun Gro® Horticulture, Massachusetts, USA) and forest soil collected from the University of Victoria campus under mature *T. plicata* and *P. menziesii* trees. All pots were co-cultured, containing one seedling from each population for a total of three seedlings per pot and twenty pots. All pots were monitored and watered with 250ml dH_2O two to three times weekly, to maintain moist soil conditions. Pots were inoculated

four times to encourage mycorrhizal colonization. 2L of the remaining forest soil was mixed with dH_2O to create 5L of slurry. The slurry was strained through a 2mm mesh sieve to remove debris, then added equally to each pot.

2.4 Non-Mycorrhizal-Aeroponic Treatment

Ten seedlings from each of the three populations were selected for the non-mycorrhizal-aeroponic treatment at the beginning of the study period. Excess soil was removed, and roots were rinsed in tap water before being transferred to 1L mason jars. Seedlings from each population were placed in the jars in pairs, for a total of five jars per population. The jars were filled with 800mL nutrient solution, wrapped in aluminum foil to protect against light, and aerated. Nutrient solution was completely replaced every seven days. With each nutrient replacement, roots were dipped in a 0.5% H_2O_2 solution for 5 seconds to discourage mycorrhizae and protect against microbial growth. After three weeks, the plants were transferred to the aeroponics buckets. Plants were grown in aeroponics for an additional fourteen weeks before being droughted, which was initiated by shutdown of the aeroponics equipment. Twenty-four hours after shutdown, a representative root tip sample was collected from each population in each bucket and stored at 4℃ for later inspection for mycorrhizal colonization and aeroponic reservoirs were emptied. Lids were lifted ~1cm to reduce humidity after 72hrs.

2.5 Mycorrhizal-Aeroponic Treatment

Ten pots of seedlings grown initially in soil using the described protocol were selected for the mycorrhizal-aeroponic treatment after seventeen weeks of growth. Seedlings were carefully separated, and soil was removed from the roots before they were rinsed briefly in tap

water. The seedlings were transferred to the aeroponic system to grow for an additional two weeks before being droughted. Immediately after initiating shutdown of the aeroponics system, reservoirs were emptied, and lids were lifted ~1cm to reduce humidity. Representative root tip samples for each population were taken from each bucket 72hrs after the start of drought conditions and stored at 4℃ for further analysis of mycorrhizal colonization.

2.6 Mycorrhizal-Soil Treatment

Ten seedlings per population were grown in soil for the duration of the study period, according to the described protocol. Prior to initiating drought conditions, pots were thoroughly soaked and left in water overnight to completely saturate the soil, then the pots were left to dry. After the experiment was complete, the pots were thoroughly soaked and left in water overnight to re-saturate the soil. Soil was removed and plants were separated to allow for root tip collection. Roots were thoroughly rinsed in tap water. A representative root tip for each population from each pot was taken and stored at 4℃ for later analysis of mycorrhizal colonization.

2.7 Droughting Procedure

Seedlings in each treatment were subject to extreme drought. Assessment of drought stress was conducted by measuring chlorophyll fluorescence, gas exchange, stem water potential, and dry biomass. A single branchlet from each seedling was marked for repeat measurements of chlorophyll fluorescence, and gas exchange.

Chlorophyll fluorescence measurements assessing quantum yield (Y(II)) were performed on all seedlings 4-8 times in an 8hr period (non-mycorrhizal-aeroponic treatment), 3-4 times (mycorrhizal-aeroponic treatment), and 1-2 times (mycorrhizal-soil treatment) using an OS1P Chlorophyll Fluorometer (Opti-Sciences Inc., New Hampshire, USA). These measurements were continued until a majority of the samples had a measured stem water potential < -2.5MPa. An additional reading was taken daily for 48hrs after the stem water potential threshold was reached.

To measure stem water potential, three random shoots from each population were selected close to the root collar, then a pressure bomb (Hoskin Scientific, British Columbia, Canada) was used to determine tension in the water column in the xylem tissue. Water potential measurements were taken at the beginning of each experiment, and at regular intervals thereafter following a sufficient decline in Y(II).

Gas exchange (evapotranspiration and CO_2 assimilation) was assessed using a LI6800 Portable Photosynthesis machine (LI-COR Biosciences, Nebraska, USA) on all seedlings. These parameters were assessed at the beginning of each experiment, as well as when Y(II) had sufficiently declined in a majority of the seedlings (Y(II)~0.600-0.650, Y(II)~0.500-0.550). Marked branchlets were removed and leaf area was determined using a LI3100 Leaf Area Meter (LICOR Biosciences, Nebraska, USA) after each experiment.

Roots and shoots were harvested and separated for measurement of dry biomass, hereafter referred to as biomass, at the end of the drought treatments. Roots and shoots were dried at 62°C for 48 hrs in a UM600 drying oven prior to weighing (Memmert GMbH + Co KG, Schwabach, Germany).

2.8 Colonization Analysis

To assess mycorrhizal colonization for each experiment, representative root tips from each population were bulked and cleared in 10% KOH at room temperature for forty-eight hours. Roots were rinsed briefly in dH_2O before being transferred to 0.05% w/v trypan blue in lactoglycerol for twenty-four hours. This follows a modified protocol based on work by Bevage (1968) and Phillips and Hayman (1970). Roots were allowed to sit in lactoglycerol to remove excess stain before each sample was assessed for colonization with dissection and compound microscopes to confirm the presence of fungal structures. Each sample was rated on a scale of 0 (no colonization) to 5 (fully colonized).

2.9 Statistical Analysis

The effect of population was tested for its effect on all measured parameters in separate analyses for each treatment. As treatments were conducted at different times, the effect of treatment could not be compared, statistically. All statistical analyses were performed using SAS software ©2022. Significance was set at $\alpha = 0.05$ and all statistically significant results were further analyzed with a Least Significant Difference means test. A GLM procedure was used to analyze the non-mycorrhizal treatment data to account for uneven sample size. An ANOVA procedure was used to analyze the mycorrhizal and soil treatments. Chlorophyll fluorescence data was analyzed using a Repeated Measures ANOVA. A one-way ANOVA was used to analyze gas exchange, assimilation, dry weight biomass for roots and shoots, and root:shoot (R:S) biomass ratios. All stem water potential data was analyzed using a GLM to account for fewer measurements.

3. Results

3.1 Non-Mycorrhizal-Aeroponic Results

3.1.1 Quantum Yield

. Chlorophyll fluorescence measurements showed differences in rates of decline in quantum yield between *P. contorta* and the two populations of *T. plicata*. Both populations of *T. plicata* declined more quickly than *P. contorta.* The experiment was considered complete on Day 7, after two of the three populations of seedlings had Y(II) values < 0.400. In this experiment, the first two populations to fall below Y(II) = 0.400 were both populations of *T. plicata*. Appreciable change in Y(II) values were not observed for any of the seedlings until > 31 hours after drought conditions had been initiated (Figure 3A). After 31 hours, mean Y(II) values began to decline and by the end of the experiment, Y(II) was significantly lower in the two *T. plicata* populations than the *P. contorta* population ($p = 0.05$, Figure 3B).

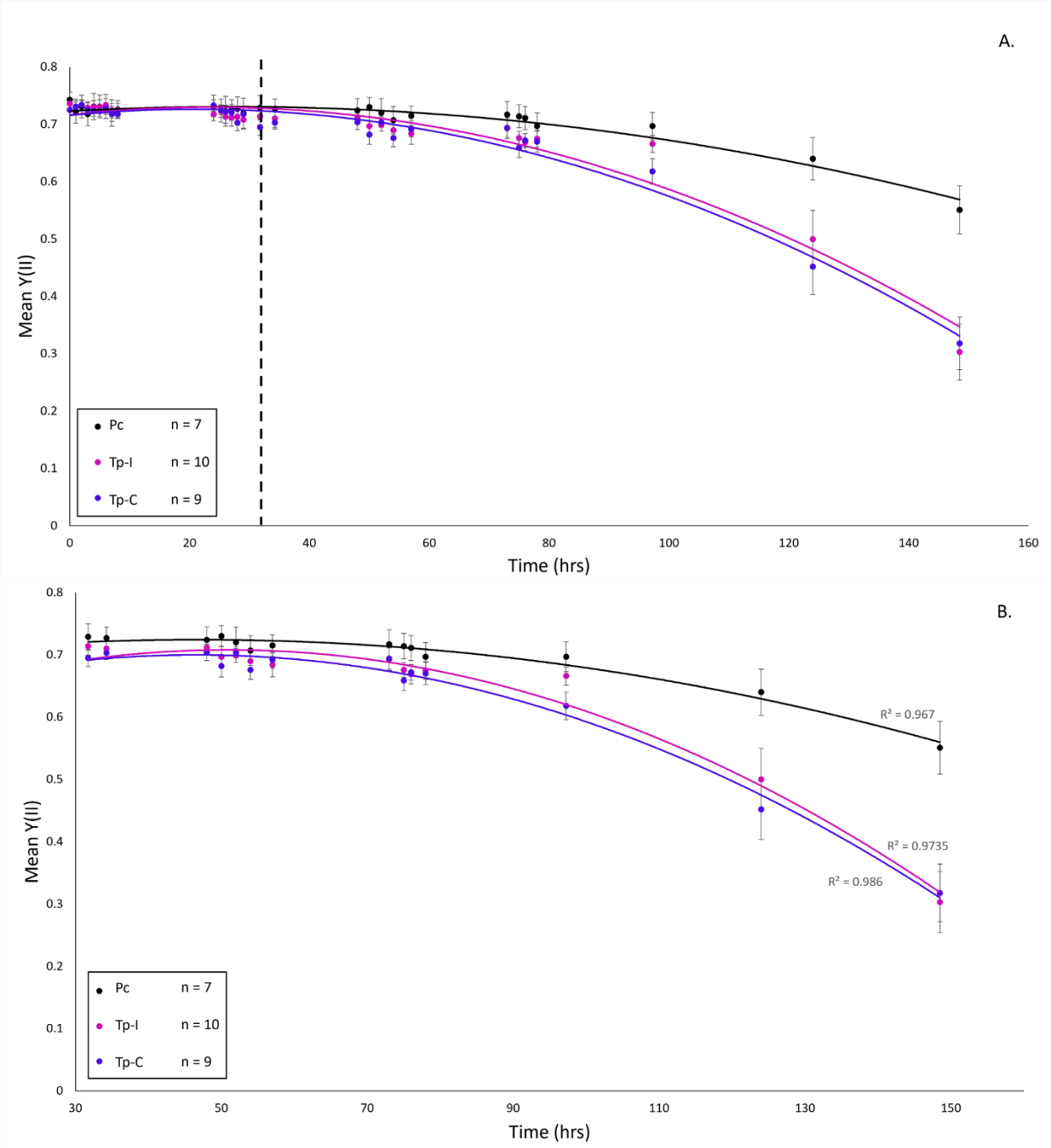

Figure 3. A. Mean quantum yield (Y(II)) ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings grown in the non-mycorrhizal-aeroponic treatment. Appreciable change in Y(II) values did not occur until 31hrs after the start of the experiment, as indicated by the dashed line. **B.** Y(II) values ± SE from 31hrs after the initiation of drought until the end of the experiment.

3.1.2 Gas Exchange

Evapotranspiration rates significantly differed between the three populations at all three time points measured (Day 2: $p = 0.008$, Day 3: $p < 0.02$, Day 5: $p < 0.04$, Figure 4A). A significant effect of replicate was found on Day 2 ($p = 0.008$). On Day 2, the rate of evapotranspiration in interior *T. plicata* was significantly lower than either *P. contorta* or coastal *T. plicata* ($p < 0.05$). On Day 3 and Day 5, evapotranspiration in the *P. contorta* population was significantly higher than either of the *T. plicata* populations ($p < 0.05$). Evapotranspiration rates in both *T. plicata* populations were similar on Day 3 and Day 5, and were not significantly different ($p > 0.05$).

Mean CO_2 assimilation rates were significantly different at the first two assessments (Day 2: $p < 0.03$, Day 3: $p < 0.0001$), with a noted interaction with replicate on both days ($p < 0.001$). By Day 5, CO_2 assimilation rates were similar over all three populations and replicate did not have an effect (Figure 4B). On Day 2, CO_2 assimilation was significantly lower in the interior *T. plicata* population compared to *P. contorta* ($p < 0.05$). On Day 3, CO_2 assimilation was not significantly different between either *T. plicata* populations ($p > 0.05$), however, both *T. plicata* populations had significantly lower rates of CO_2 assimilation compared to P. contorta ($p < 0.05$).

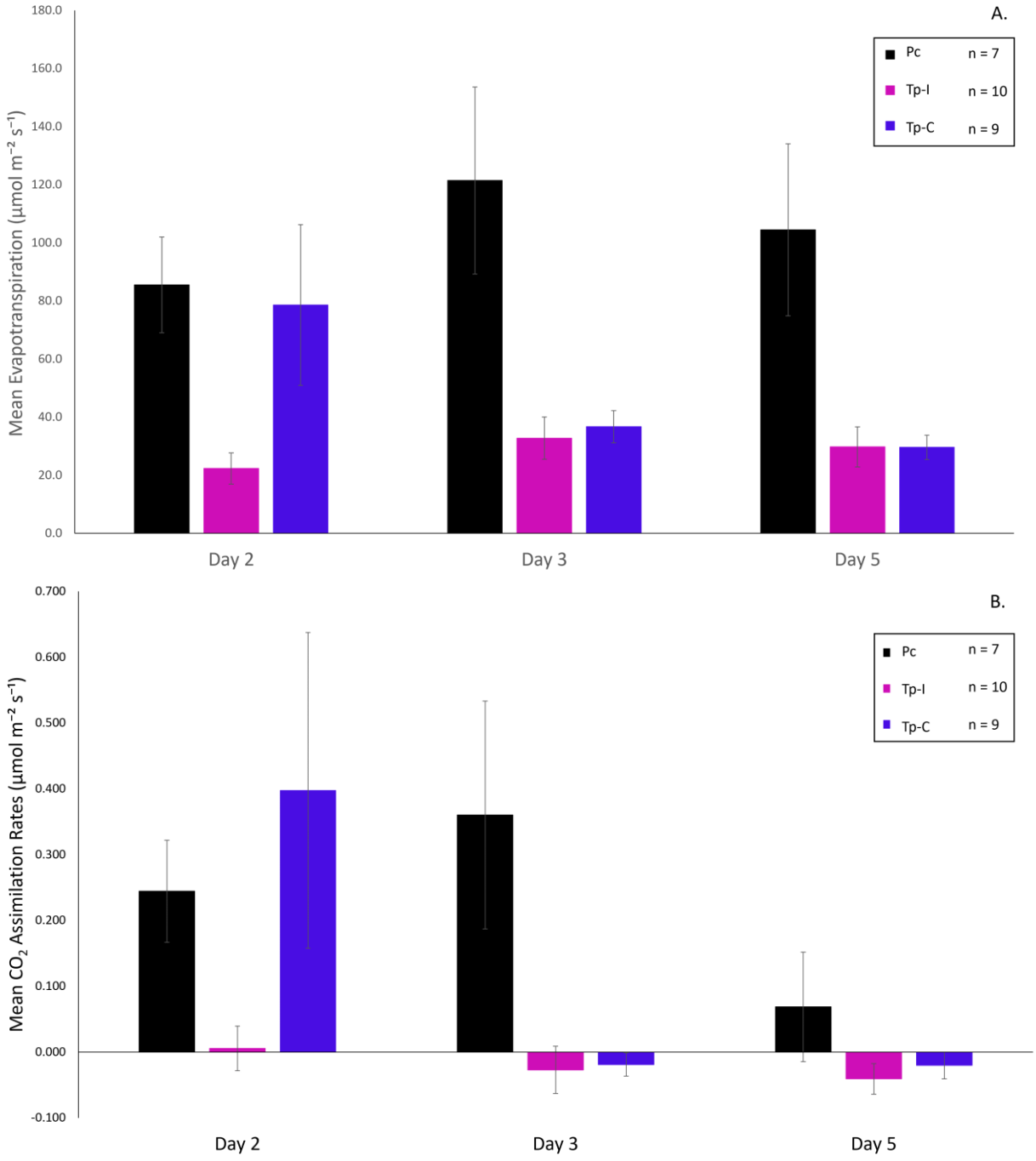

Figure 4. Gas exchange for *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings over time after drought conditions had been induced in the non-mycorrhizal-aeroponic treatment. **A.** Mean evapotranspiration (E) rates ± SE. **B.** Mean CO_2 assimilation (A) rates ± SE.

3.1.3 Stem Water Potential

Stem water potential differed significantly between species on all assessment dates ($p < 0.001$). *P. contorta* showed less negative water potential consistently compared to both populations of *T. plicata* (Figure 5). Stem water potential was not found to be significantly different between the two populations of *T. plicata* ($p > 0.05$).

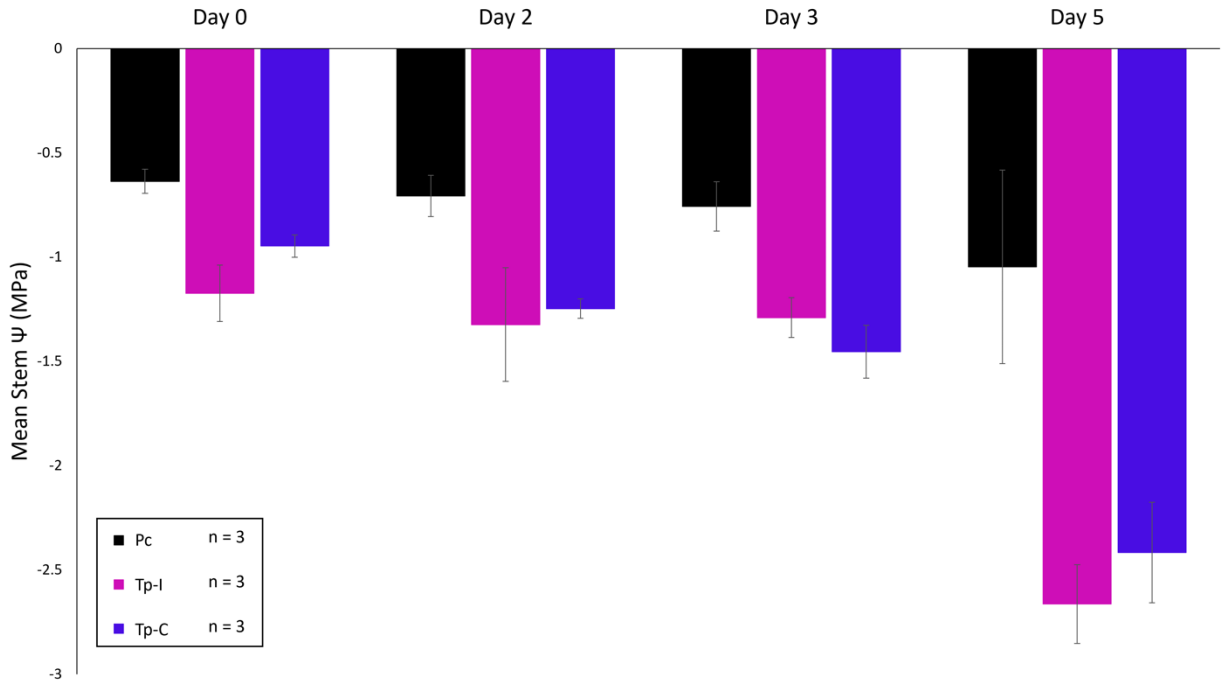

Figure 5. Mean stem water potential ± SE over time after droughting conditions were initiated in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings in the non-mycorrhizal-aeroponic treatment.

3.1.4 Biomass

Shoot biomass differed significantly between the three populations ($p < 0.001$, Figure 6). Root biomass was not significantly different between the three populations ($p = 0.12$). Total biomass was significantly different in *P. contorta* seedlings compared to both populations of *T. plicata* seedlings ($p < 0.001$). R:S ratios were significantly different between *P. contorta*

seedlings and the two *T. plicata* seedlings ($p < 0.001$), but no significant difference was found between the two *T. plicata* populations ($p > 0.05$). Mean R:S ratios were 2.52 ± 1.68 for *P. contorta*, 0.90 ± 0.38 for interior *T. plicata*, and 0.66 ± 0.23 for coastal *T. plicata*.

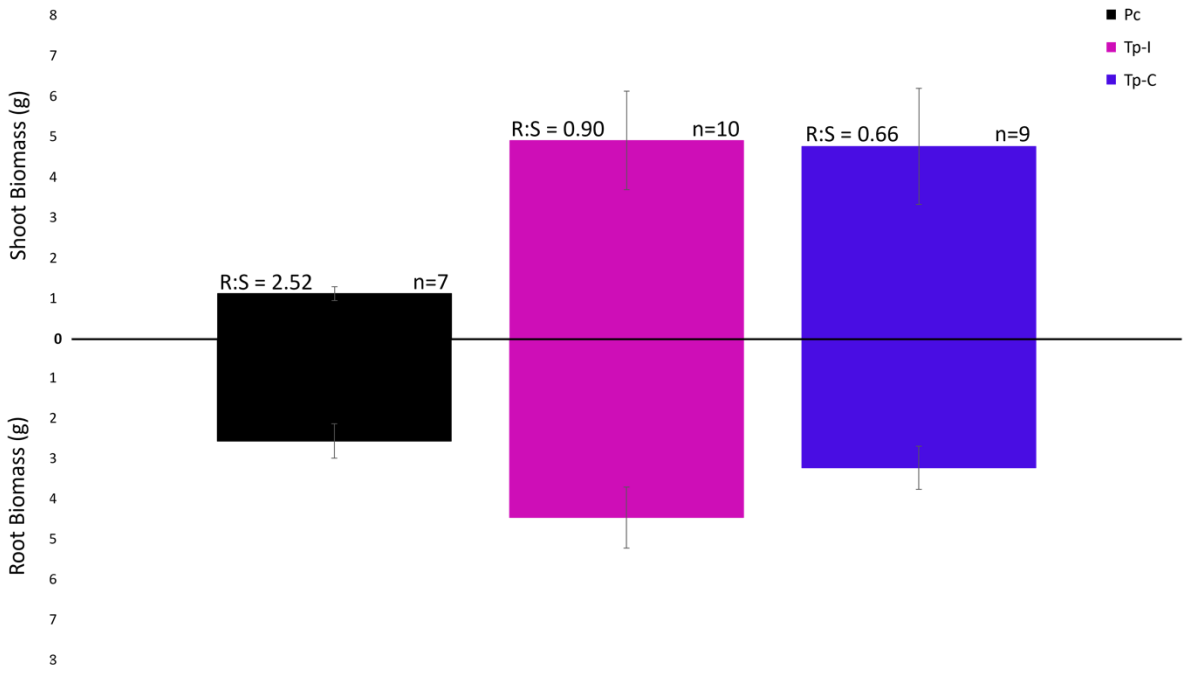

Figure 6. Mean dry biomass for roots and shoots ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* after the completion of the non-mycorrhizal-aeroponic treatment.

3.2 Mycorrhizal-Aeroponic Results

3.2.1 Quantum Yield

Quantum yield declined steadily for all three populations and was found to be significantly different between them over time ($p < 0.001$). Seedlings from both populations of *T. plicata* were found to be below Y(II) = 0.400 on Day 6 of the experiment, before the population

of *P. contorta* seedlings. *P. contorta* showed slower decline in quantum yield values than both *T. plicata* populations (Figure 7).

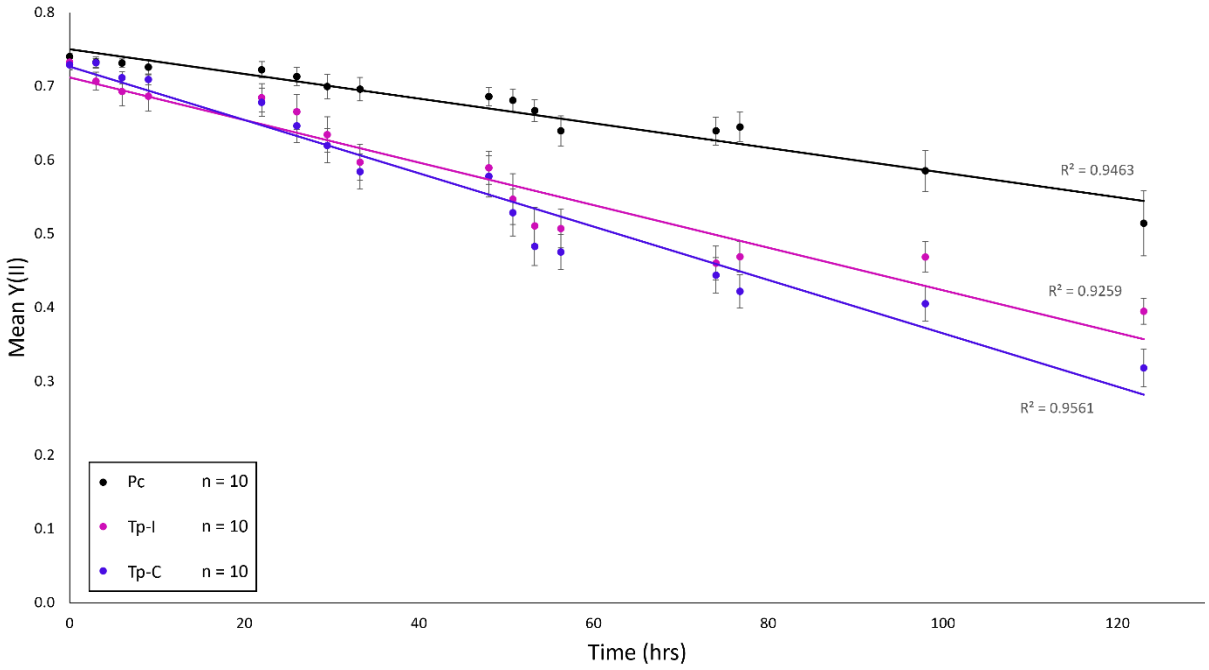

Figure 7. Mean quantum yield (Y(II)) values ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I) and coastal *T. plicata* (Tp-C) over time, after droughting conditions had been initiated in the mycorrhizal-aeroponics treatment.

3.2.2 Gas Exchange

Evapotranspiration rates among the three populations declined over the duration of the experiment. In all cases, *P. contorta* exhibited the highest rates of evapotranspiration (Figure 8A). On Day 0 at the start of the experiment, this trend was found to be statistically significant ($p < 0.005$) between *P. contorta* and the two *T. plicata* populations. No significant difference was observed between the two *T. plicata* populations ($p > 0.05$). On Day 2, no significant difference in evapotranspiration rates was found among any of the populations ($p = 0.139$). Similarly, on

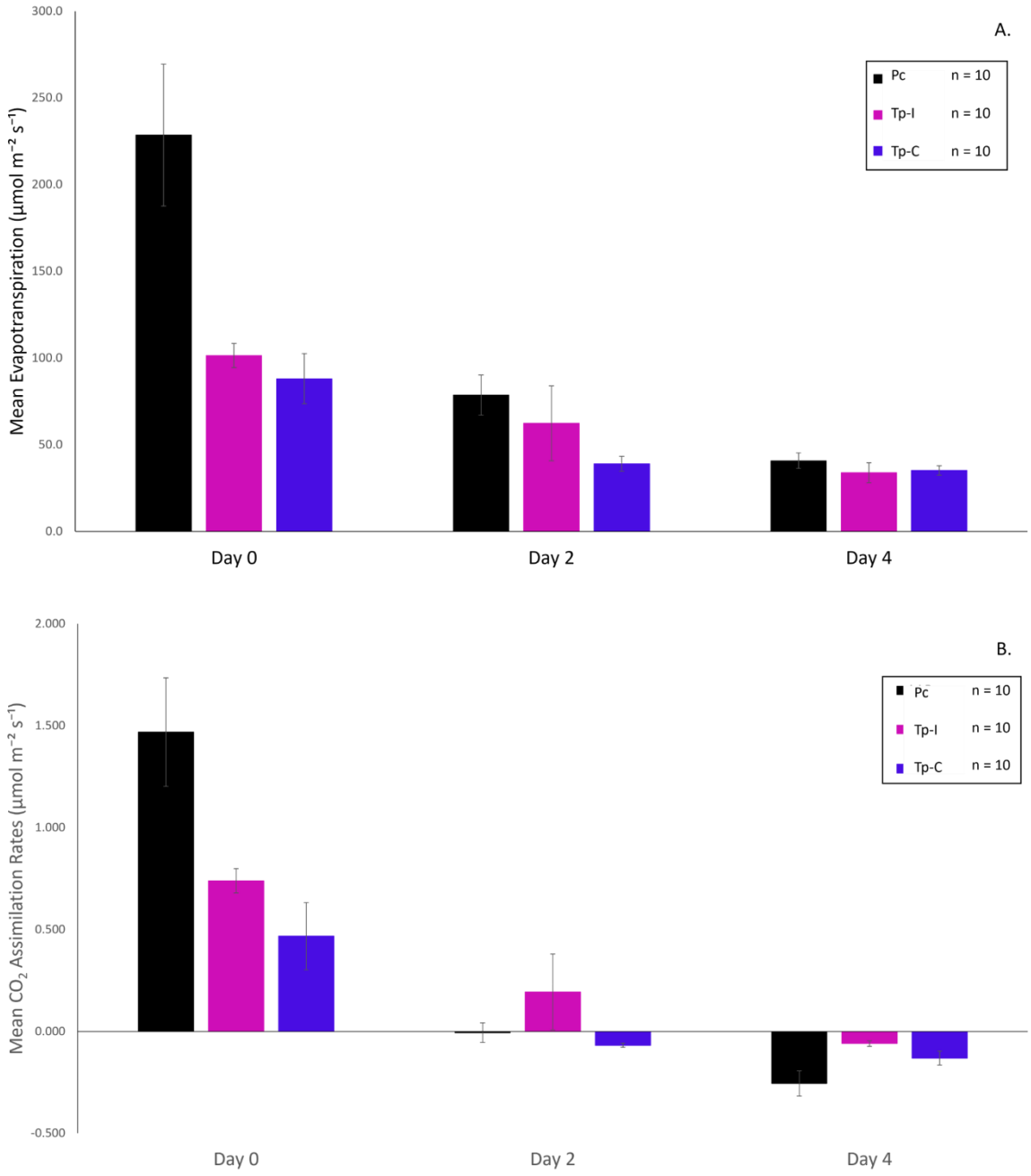

Figure 8. Gas exchange in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings over time after drought conditions had been induced in the mycorrhizal-aeroponics treatment. **A.** Mean evapotranspiration (E) rates ± SE. **B.** Mean CO_2 assimilation (A) rates ± SE.

Day 4, evapotranspiration rates were shown to be not statistically significant among populations ($p = 0.454$).

CO_2 assimilation rates also declined over time (Figure 8B). At the start of the experiment, *P. contorta* had significantly higher rates of CO_2 assimilation than either population of *T. plicata* ($p < 0.01$). No significant difference in CO_2 assimilation rates was evident between any of the populations ($p = 0.189$). By Day 4, all seedlings were respiring and there were no significant differences between any of the populations ($p = 0.063$).

3.2.3 Stem Water Potential

Stem water potential was significantly different between the three populations over time ($p < 0.001$). Coastal *T. plicata* consistently had a significantly lower stem water potential than both *P. contorta* and interior *T. plicata* ($p < 0.05$, Figure 9), but no significant difference was found between interior *T. plicata* and *P. contorta* over the course of the experiment ($p > 0.05$).

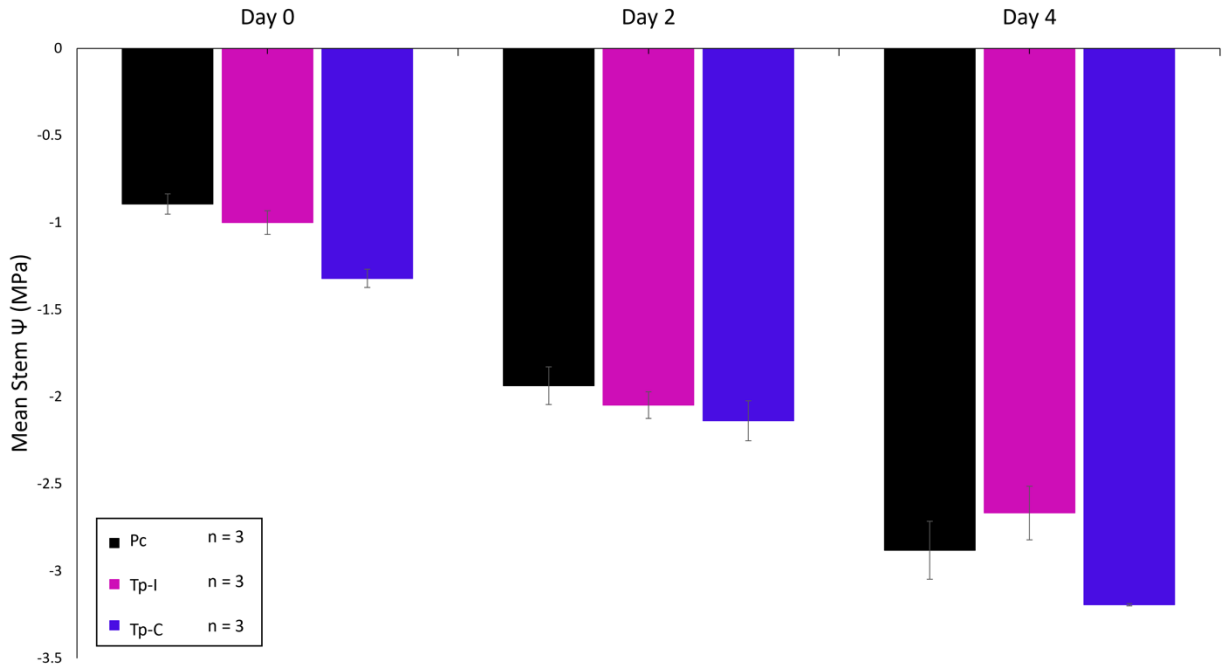

Figure 9. Mean stem water potential (Ψ) ± SE over time after droughting conditions were initiated in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings in the mycorrhizal-aeroponic treatment.

3.2.4 Biomass

Shoot biomass was not significantly different between the three populations ($p = 0.30$, Figure 10). A significant difference was found between root biomass for *P. contorta* and both *T. plicata* populations ($p < 0.001$), but no significant difference was found between the two *T. plicata* populations ($p > 0.05$). Total biomass was also significantly different between the *P. contorta* seedlings and the two populations of *T. plicata* seedlings ($p < 0.005$), but not significantly different between the two populations of *T. plicata* seedlings ($p > 0.05$). R:S ratios

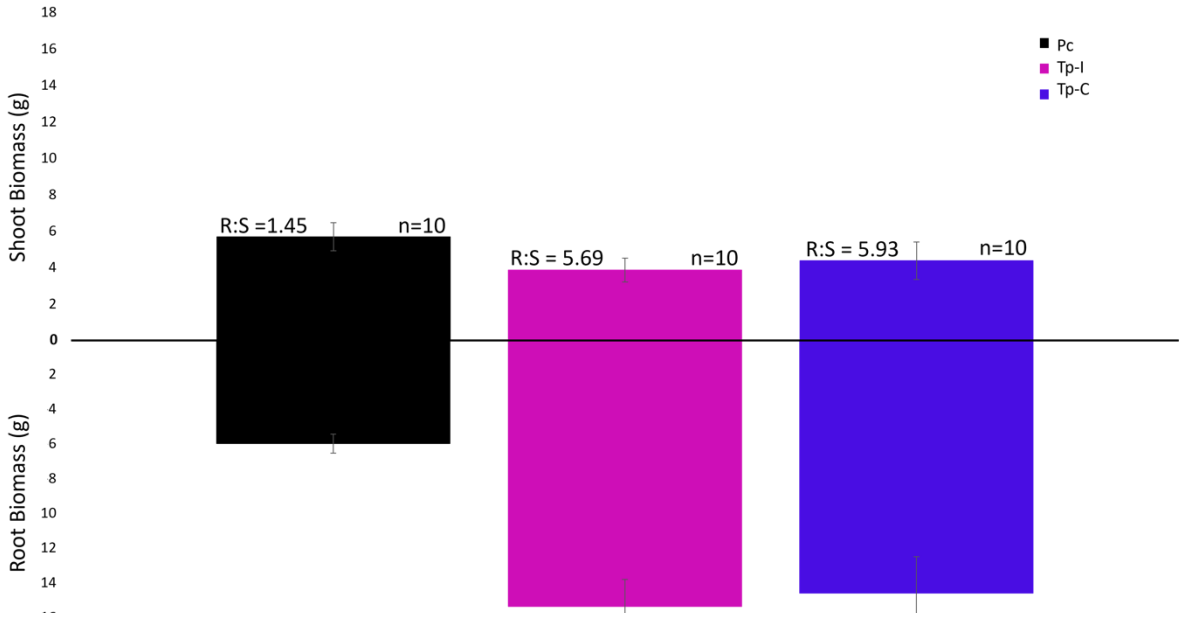

Figure 10. Mean dry biomass for roots and shoots ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* after the completion of the mycorrhizal-aeroponic drought experiment.

were significantly different between the three populations ($p = 0.02$). A significant difference was found for R:S ratios between *P. contorta* and both *T. plicata* populations, but no significant difference was found between the two *T. plicata* populations ($p > 0.05$). Mean R:S ratios were 1.45 ± 1.17 for *P. contorta*, 5.69 ± 4.36 for interior *T. plicata*, and 5.93 ± 4.89 for coastal *T. plicata*.

3.3 Mycorrhizal-Soil Results

3.3.1 Quantum Yield

Chlorophyll fluorescence measurements showed differences in rates of decline in quantum yield between *P. contorta* and the two populations of *T. plicata* growing in soil. Both populations of *T. plicata* showed a faster decline of Y(II) than *P. contorta* and by Day 33 of the experiment, both populations of *T. plicata* had dropped below Y(II) = 0.400. No appreciable change was observed in quantum yield in any of the populations prior to 23 days after the last water application to the soil (Figure 11A). After 23 days, *P. contorta* and the two populations of *T. plicata* began to diverge in their rates of Y(II) decline (Figure 11B). Overall, the rate of decline in Y(II) was significant ($p < 0.02$). This rate varied significantly between individual replicates, but the differences between populations did not become significant until Day 29 ($p = 0.032$). The differences between replicates continued to increase in significance until the end of the experiment.

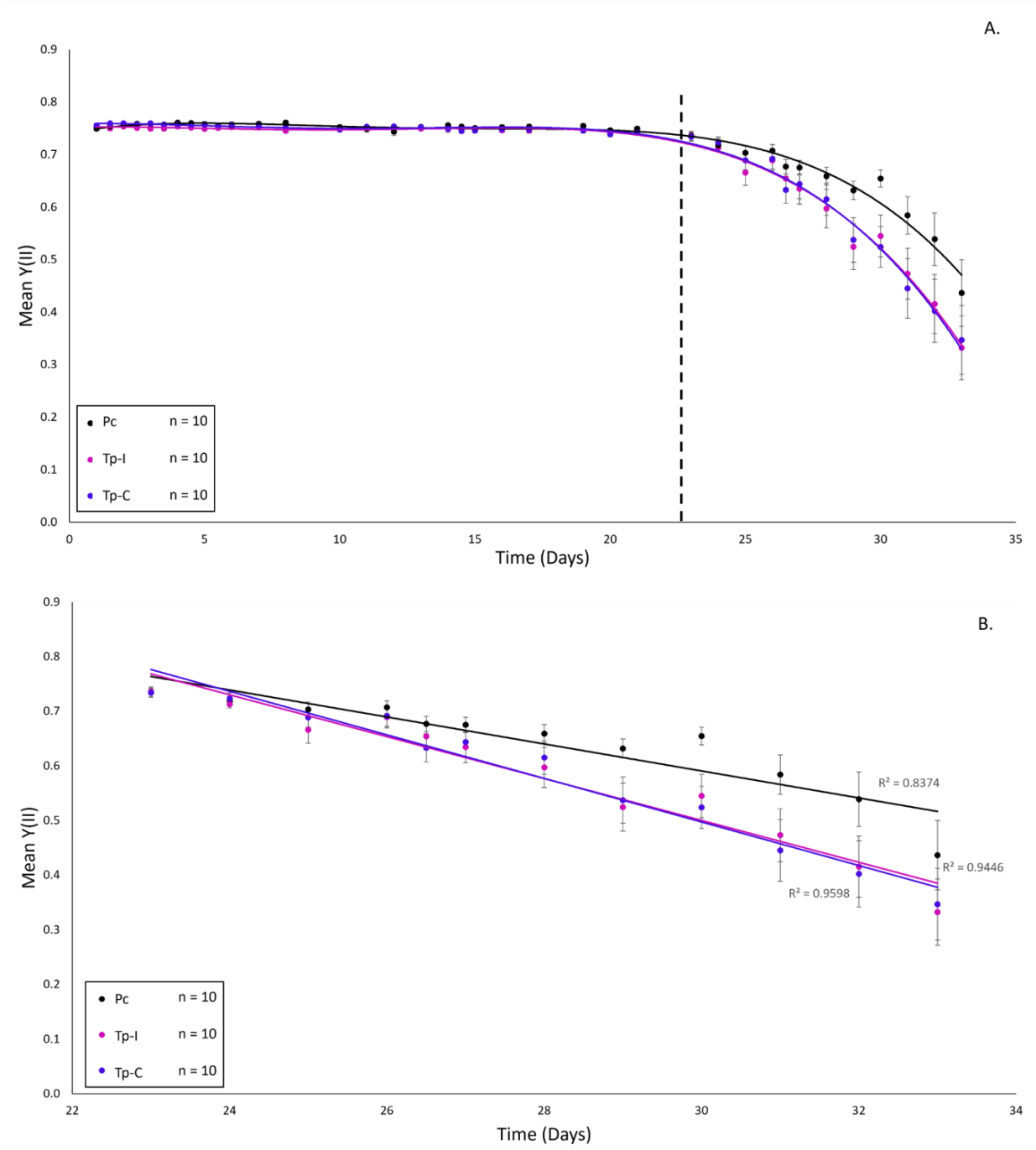

Figure 11. Mean quantum yield (Y(II)) ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings grown in the mycorrhizal-soil treatment. **A.** There were no significant changes observed in Y(II) until Day 23 of the experiment, indicated by the dashed line. **B.** Y(II) values ± SE from Day 23 after the initiation of drought until the end of the experiment.

3.3.2 Gas Exchange

Evapotranspiration rates of the three populations declined over the duration of the experiment (Figure 12A). Evapotranspiration rates were similar across the three populations and did not significantly differ on any measurement date (Day 0: $p = 0.0796$, Day 24: $p = 0.5867$, Day 30: $p = 0.7881$). CO_2 assimilation rates were also similar across the three populations (Figure 12B) and did not significantly differ on any measurement date (Day 0: $p = 0.0657$, Day 24: $p = 0.4652$, Day 30: $p = 0.3061$).

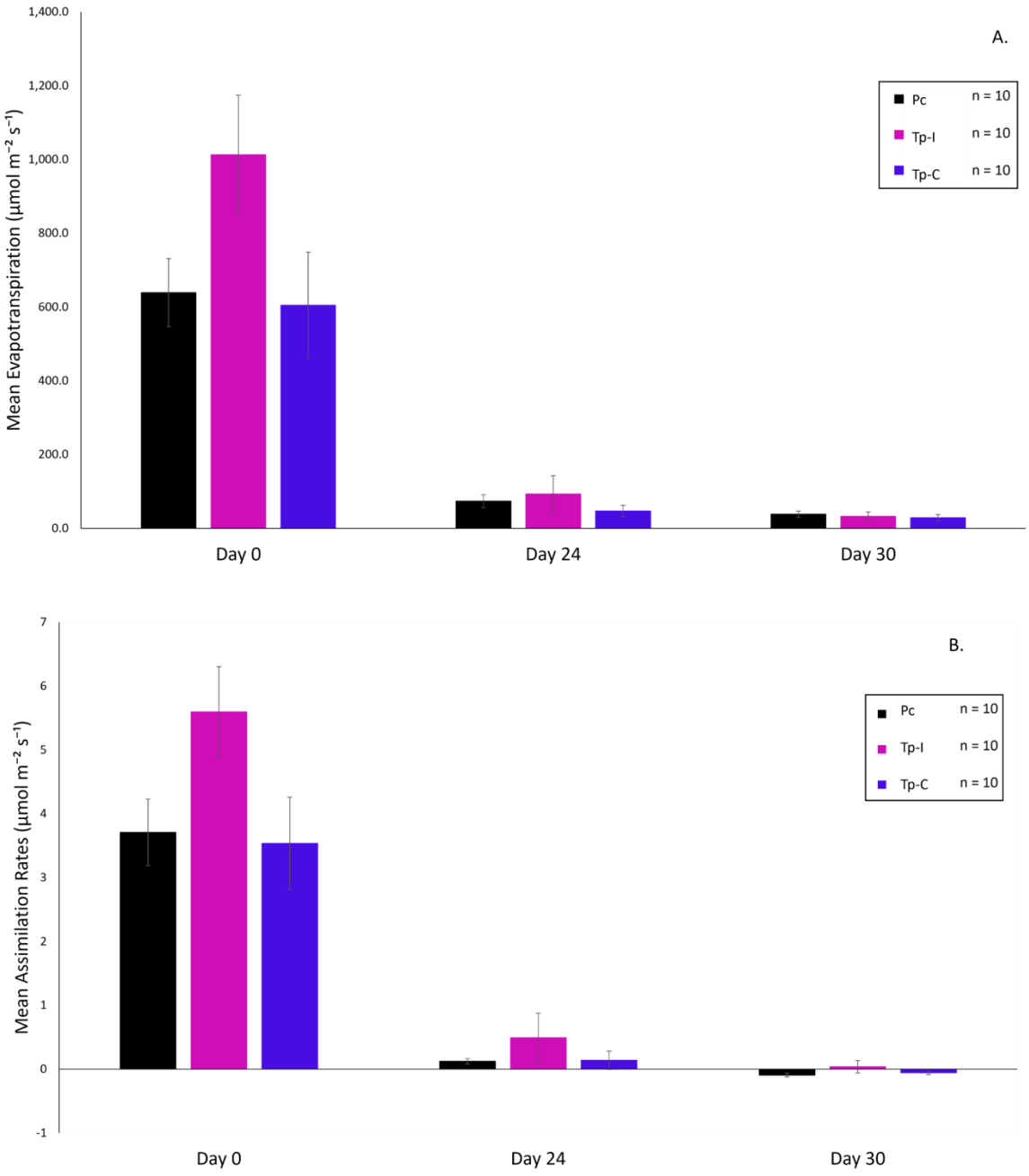

Figure 12. Gas exchange parameters for *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings over time after drought conditions had been induced in the mycorrhizal-soil treatment. **A.** Mean evapotranspiration (E) rates ± SE. **B.** Mean CO_2 assimilation (A) rates ± SE.

3.3.3 Stem Water Potential

Stem water potential was significantly different between *P. contorta* and coastal *T. plicata* over the course of the experiment ($p < 0.001$, Figure 13), with *P. contorta* being less drought stressed. A significant difference was not observed between *P. contorta* and interior *T. plicata* over the course of the experiment ($p > 0.05$). By the end of the experiment, both populations of *T. plicata* were not significantly different from each other ($p > 0.05$).

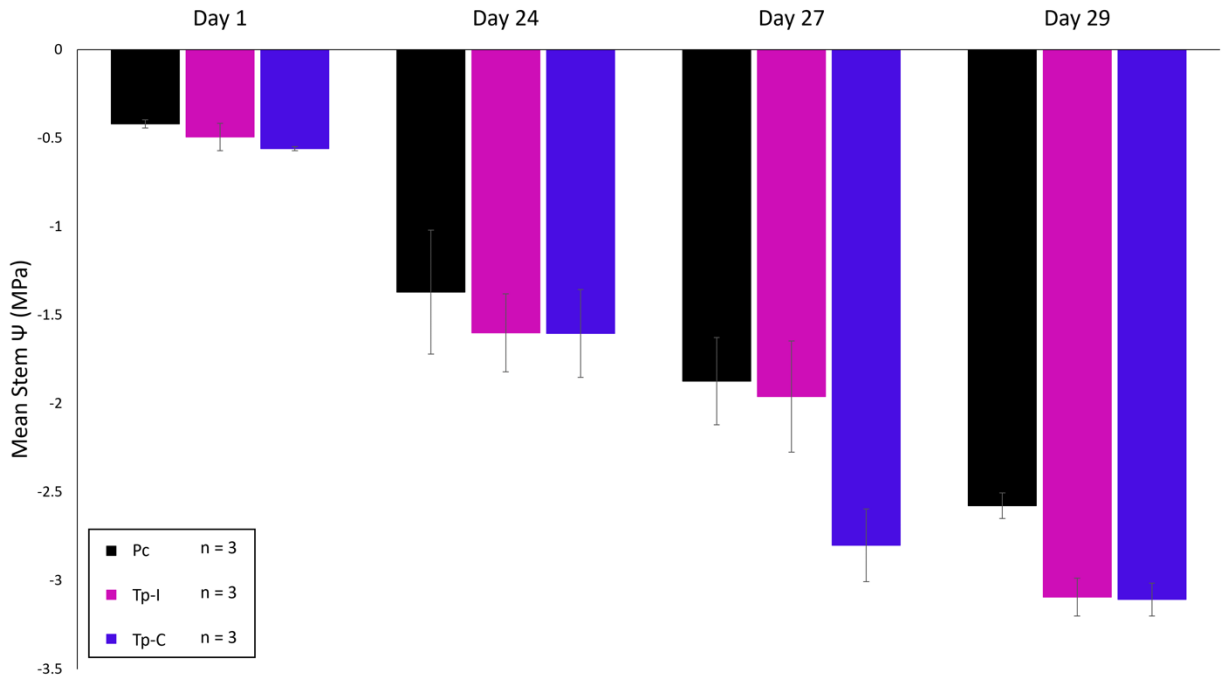

Figure 13. Mean stem water potential (Ψ) ± SE over time after droughting conditions were initiated in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* (Tp-C) seedlings in the mycorrhizal-soil treatment.

3.3.4 Biomass

Shoot biomass was not significantly different between the three populations ($p = 0.47$, Figure 14). Root biomass was significantly different between *P. contorta* and both *T. plicata* populations ($p < 0.001$), but no significant difference was found between the two *T. plicata*

populations ($p > 0.05$). Total biomass was significantly different between *P. contorta* and both *T. plicata* populations ($p < 0.009$), but the two populations of *T. plicata* were not significantly different from each other ($p > 0.05$). R:S ratios were also significantly different between the three populations ($p < 0.001$). A significant difference was found between interior *T. plicata* and both coastal *T. plicata* and *P. contorta*, but no significant difference was found between coastal *T. plicata* and *P. contorta* ($p > 0.05$). Mean R:S ratios were 0.88 ± 0.96 for *P. contorta*, 3.72 ± 2.91 for interior *T. plicata*, and 1.82 ± 1.26 for coastal *T. plicata*.

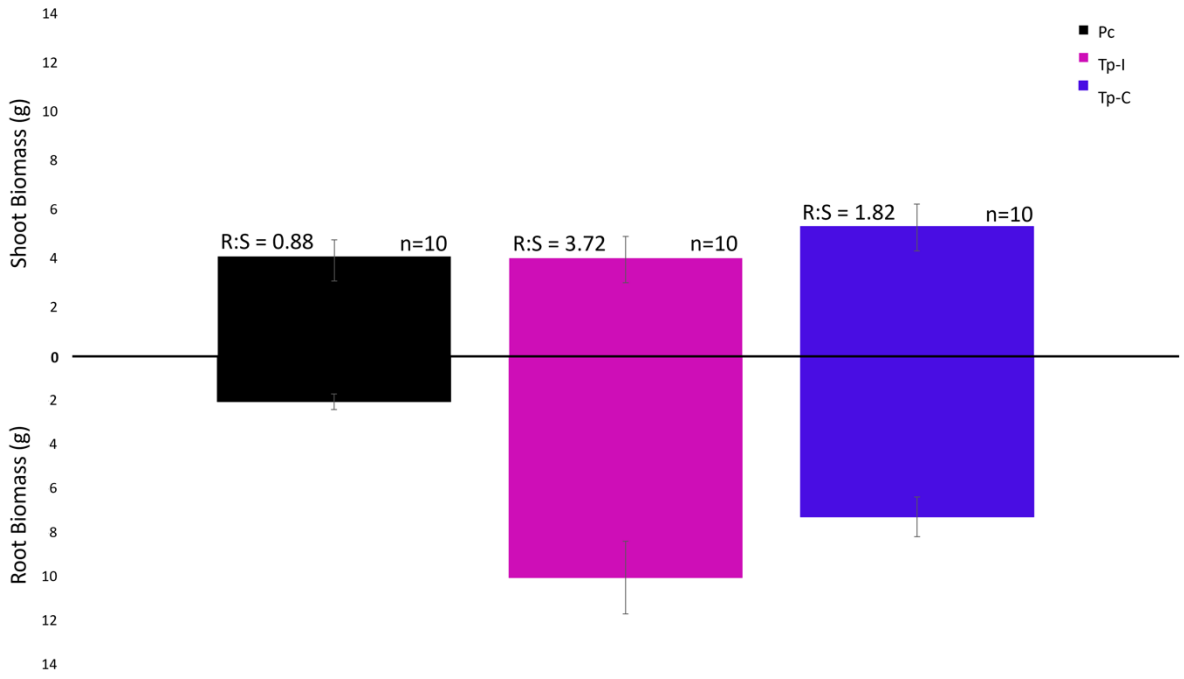

Figure 14. Mean dry biomass for roots and shoots ± SE in *Pinus contorta* (Pc), interior *Thuja plicata* (Tp-I), and coastal *T. plicata* after the completion of the mycorrhizal-soil drought experiment.

3.4 Mycorrhizal Colonization Analysis

Some mycorrhizal colonization was seen in roots from all three treatments after staining (Figure 15). Roots in the non-mycorrhizal-aeroponic treatment all showed colonization but at relatively low levels, with most samples scoring a 2 or lower out of 5. In contrast, roots in the

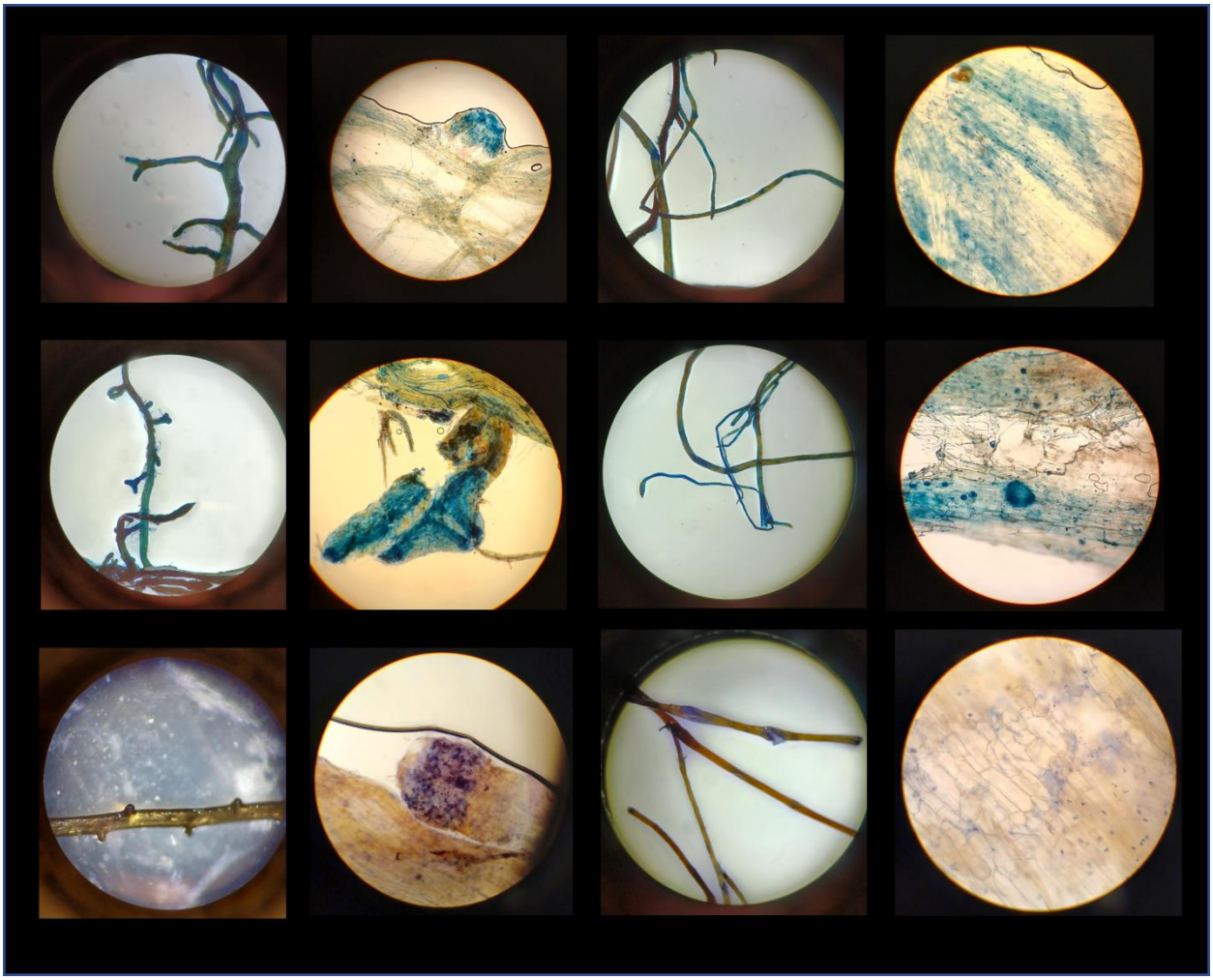

Figure 15. Representative microscopy images from staining root tips with 0.05% Trypan blue in lactoglycerol across all three treatments. Blue coloration is associated with fungal tissue; small blue dots in arbuscular mycorrhizal samples are suspected vesicles. Each sample is seen at both low and high magnifications. **Top Row – Mycorrhizal-soil treatment (L-R):** *Pinus contorta* roots at 2X magnification, showing branching associated with ectomycorrhizal colonization. *P. contorta* roots at 20X magnification showing a newly colonized branch. *Thuja plicata* roots at 1X magnification, showing that roots are well colonized. *T. plicata* roots at 20X magnification. **Middle Row – Mycorrhizal-aeroponic treatment (L-R):** *P. contorta* roots at 1X magnification. Roots are thoroughly colonized and showing branching pattern associated with ectomycorrhizae. *P. contorta* roots at 20X magnification showing an ectomycorrhizal Hartig net. *T. plicata* roots at 1X magnification. Roots are almost completely colonized. *T. plicata* roots at 20X magnification. **Bottom Row – Non-mycorrhizal-aeroponic treatment (L-R):** *P. contorta* roots at 4X magnification. Branching pattern shows possible colonization with ectomycorrhizae with limited structural development. *P. contorta* root at 40X magnification. Shown in blue is a Hartig net structure. *T. plicata* roots at 0.8X magnification with minimal colonization. *T. plicata* roots at 40X magnification.

mycorrhizal-aeroponic and mycorrhizal-soil treatments both scored very high on the ranking

scale, with most roots appearing heavily colonized and scoring 4 to 5 out of 5.

4. Discussion

The objective of this study was to determine whether ectomycorrhizae can improve drought tolerance to a greater extent than arbuscular mycorrhizae. It was predicted that *P. contorta* would fare better in drought conditions across all treatments, but that in both the mycorrhizal-aeroponic treatment and the mycorrhizal-soil treatment, the difference between both *T. plicata* populations and *P. contorta* would be more pronounced. It was also predicted that the two populations of *T. plicata* would differ in their drought responses based on their climate of origin.

Quantum yield was used to assess the health of photosystem II, as it indicates the amount of light energy used in photosynthesis when plants have already been exposed to light, such as during daylight. Stem water potential was used to directly assess the tension on the water column in the xylem tissue. Gas exchange was used to assess drought stress symptoms such as closure of stomata, as it is a measure of water loss through evapotranspiration and CO_2 uptake.

4.1 Species Effects

It is evident across all three treatments that species was a significant factor in the results. *P. contorta* seedlings were considerably less stressed than either population of *T. plicata* in all treatments. A study conducted by Montwe and colleagues (2015) showed that *P. contorta* is well adapted to drought across several ecozones and that adaptation to drought was well correlated with where the population originated. These findings agree with the results presented here and support the prediction that *P. contorta* would be tolerant of drought conditions in all treatments, despite originating in a coastal climate.

Challenging the prediction that *T. plicata* populations would differ in response to drought, both populations of *T. plicata* showed surprisingly little variation in their water stress responses, regardless of treatment. A study completed by Fan and colleagues (2008) supports the lack of variation in drought responses in *T. plicata* populations. Fan *et al* (2008) found that location-specific adaptation in *T. plicata* is more likely to be morphological in nature, and showed that most populations of *T. plicata* did not exhibit significant variation in water stress responses, regardless of the precipitation patterns found in their native ecosystem. However, another study completed by Grossnickle and Russell (2010), found that *T. plicata* did show variation in drought responses according to parental precipitation patterns, suggesting that the response of *T. plicata* to water stress is complex. In both studies, it is unclear how the role of mycorrhizae may have affected the outcomes, but the trends are similar to the results in this study. Interestingly, in the current study, a location effect was seen in stem water potential values for both the mycorrhizal-aeroponic and mycorrhizal-soil treatments. In both treatments, coastal *T. plicata* experienced significantly lower water potential values than interior *T. plicata* over the duration of the experiment. This did not appear to significantly affect gas exchange or photosynthesis. This study had variation in drying times across treatments, so no comparisons may be drawn here. However, further research in this area may provide valuable insights into how mycorrhizae might differ in their mediation of drought tolerance in this species.

4.2 Mycorrhizal Influences

Determining the degree of effect of mycorrhizal type on drought tolerance is complex. In the non-mycorrhizal aeroponic treatment, the seedlings across the populations showed a non-linear decline in Y(II). Contrasting with this, the seedlings across all the populations in the mycorrhizal-aeroponic treatment showed a linear decline in Y(II). This would suggest that both

types of mycorrhizae mitigated some of the effects of drought stress on these seedlings. Both types of mycorrhizae are well known to mediate responses to a wide variety of stresses, including drought, and the findings in the current study are consistent with research conducted on other tree species.

4.2.1 Effects of Arbuscular Mycorrhizae

Arbuscular mycorrhizae have been shown to utilize many mechanisms in reducing water stress in their host plants. A study conducted on *Dipteryx alata Vogel* showed that arbuscular mycorrhizae improved drought tolerance in that species (Jesus et al, 2022). Their results focused on parameters involved in photosynthetic metabolism and showed a clear improvement in drought responses for inoculated seedlings, which is similar to the findings presented here. AM associations are widespread, and it is reasonable to consider that some of the mechanisms they use to mediate drought tolerance in plants are also conserved across species. Research completed by Al-Arjani and associates (2020) showed that arbuscular mycorrhizae could alter hormone production in the host plant under drought conditions, including abscisic acid, and increase antioxidant enzyme activity. The results showed that AM colonization significantly improved drought responses in the host plant, which is consistent with the results presented here, though the mechanism by which this occurred in the present study is unknown.

4.2.2 Effects of Ectomycorrhizae

Ectomycorrhizae have been shown to improve drought tolerance primarily as a result of increased access to nutrients. A study conducted by Li and colleagues (2021) showed the improvement of drought responses in ectomycorrhizal *Quercus acutissima*, through increased Ca^{2+} uptake. This study shows a similar trend for enhanced drought tolerance resulting from

ECM colonization, using Y(II) as the measure, but the mechanism for that trend may differ from what is seen here. Another investigation, completed by Agathokleous and colleagues (2022) on *Larix kaempferi* showed similar findings to the current study. They found that ECM colonization had no effect on gas exchange but did have a positive effect on water potential in drought conditions. The study also showed that drought stressed plants had improved nutrient uptake, with a significant enrichment in foliar phosphorous. Both studies support the general trends seen in the *P. contorta* seedlings in both the mycorrhizal-aeroponics treatment and the mycorrhizal-soil treatment, on the basis that both treatments seem to have improved drought responses, especially in Y(II), compared to the non-mycorrhizal-aeroponic treatment. The mycorrhizal-soil treatment took longer to decline, based on when Y(II) ~ 0.600 and the length of time it took to drop again to Y(II) ~ 0.400. In the mycorrhizal-aeroponics treatment, nutrient access is highly available during watered periods and completely unavailable after drought conditions are initiated. Because there are differences in nutrient acquisition during drought stress in these treatments, further investigation may find other mechanisms beyond nutrient uptake that could be contributing to drought tolerance.

4.3 Conclusion

This research was focused on how the increase of water stress affected *P. contorta* and *T. plicata* seedlings with and without mycorrhizal roots, and the results highlight the complexity of water stress responses in conifers. Based on these results, mycorrhizae appear to play a role in mediating drought responses in these species, though whether the type of mycorrhizae has any significant impact is less certain. There is no obvious difference to suggest that ectomycorrhizae

improve drought tolerance in their host species more than arbuscular mycorrhizae. In future studies it may also be useful to look at nutrient concentrations in drought stressed ECM colonized conifers in comparison to AM colonized conifers, as this may help to elucidate some of the differences between these two groups of fungi and their role in mitigating drought stress.

Drought is a complex natural phenomenon, and rarely exists as an isolated event. This study was limited to extreme water stress, but drought is often coupled with heat. Mycorrhizae may benefit their host plants differently with coupled stresses, and the combination of heat and drought may more accurately reflect conditions in the field. Furthermore, it is unknown how mycorrhizae may affect drought recovery in these species and varying levels of drought may also elicit a different response. The research presented here can inform future studies investigating the role of mycorrhizae in the drought response of these species, as well as the mechanisms by which this may occur. The knowledge this may provide could inform conservation and climate mitigation efforts in forests susceptible to drought, as well as forestry management practices for these two conifers.

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Supplemental

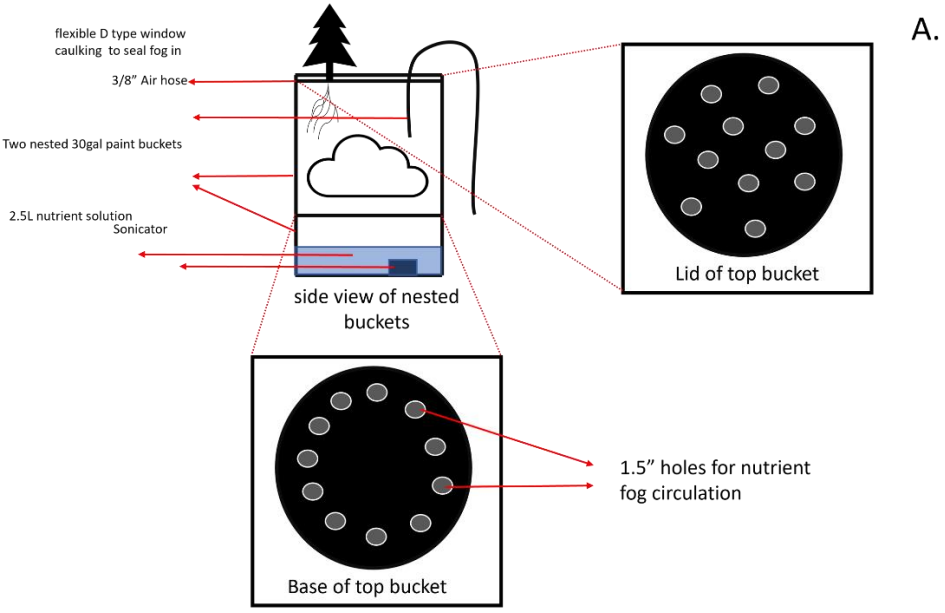

Figure S1. A. A graphic representation of the aeroponics system. Two black 30gal paint buckets are nested together, with a line of D-type flexible window caulking to seal the gap between them. Holes in the base of the top bucket allow circulation of fog while keeping the roots from hanging directly into the nutrient solution. Ten holes in the top lid keep specimens suspended, using foam or rockwool to keep them in place once they are transferred into aeroponics. The bottom bucket holds 2.5L of nutrient solution and a sonicator aerosolizes the solution into a fog. A 3/8" air hose can be attached from an air pump (not pictured) into the top bucket to keep the fog moving around the roots. Fog is timed for 3min on and 6min off using an analog cyclostat (not pictured) **B.** Three sets of nested buckets are shown during the transfer of seedlings into the system. The middle bucket is in the process of being cleaned and filled with nutrient solution in preparation for the addition of seedlings. **C.** Three sets of buckets in the growth chamber; every bucket holds 3-4 seedlings each of interior *Thuja plicata*, coastal *T. plicata*, and *Pinus contorta*. **D.** Roots are shown from the non-mycorrhizal-aeroponic treatment with new root tip growth. In the bucket, nutrient fog is visible.