# AN ABSTRACT OF THE THESIS OF

Kaitlin A. Gerber for the degree of Master of Science in Sustainable Forest Management presented on April 16, 2018.

Title: Simulated Environmental Stressors Influence Fabaceae Seedling Development

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Anthony S. Davis

Sites in need of restoration typically have one or more environmental factors that limit seedling establishment; identifying ecophysiological responses to environmental stressors can be advantageous in growing seedlings able to overcome such constraints on survival. To maximize survival after outplanting, seedlings should be grown in a manner that considers both the natural development of the species as well as the potential outplanting conditions. Black locust (*Robinia pseudoacacia* L.) and koa (*Acacia koa* A. Gray) are nitrogen-fixing trees that are used in restoring degraded ecosystems; this thesis focuses on projects aimed at providing a stronger understanding of the seedling behavior of those species in response to environmental conditions.

The first study examined how koa seedlings responded to reduced water (W), reduced phosphorus (P), and combined reduced W and P conditions. It was found that after 17 weeks, seedlings subjected to reduced W or reduced P treatments accumulated less biomass, had smaller root-collar-diameters, narrower root structures, developed less nodules, contained lower C and N contents, and lower foliar P concentrations. Combined reductions in W and P interacted such that seedlings increased their root to shoot dry biomass and developed shorter shoots. Seedlings treated with reduced W had reduced instantaneous rates of $CO_2$ assimilation, but higher

instantaneous water-use efficiency. Seedlings under reduced P treatments had a similar rate of $CO_2$ assimilation relative to those grown with adequate P, suggesting that koa is able to employ strategies to avoid physiological impairment during photosynthesis. Water stressing seedlings in an artificial environment before planting on sites low in soil moisture may physiologically cue seedlings to develop greater resistance to drought stress. Similarly, field P fertilization may enhance seedling growth rates, especially on sites with low plant-available water.

The second study evaluated if the amount of fertilizer used to grow black locust seedlings can be reduced without compromising seedling morphology through inoculating seeds with rhizobium. Seedlings were grown under different fertilizer application rates (0, 2, or 4 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$) and either inoculated with rhizobium or left uninoculated to determine the nursery growing regime that produced the largest seedlings with the greatest nodule formation. It was found that seedlings grown under 4 mg Applied Fertilizer·seedling$^{1}$·week$^{-1}$ were the largest regardless of inoculation. However, seedlings grown under 2 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$ had a comparable height and root dry mass. Inoculation and fertilizer rate impacted nodule formation such that seedlings inoculated or grown under the 2 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$ had the greatest nodule formation; this treatment combination interacted such that seedlings had comparable relative height growth and shoot dry mass compared to the seedlings grown under 4 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$. Seedlings grown under 0 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$ were stunted in growth and formed less nodules regardless of inoculation. The result indicates that nurseries should fertilize black locust seedlings, but may be able to drastically reduce the amount of fertilizer used, reducing the possibility of local surface and groundwater pollution, if seeds are inoculated with rhizobium while sowing.

Simulated Environmental Stressors Influence Fabaceae Seedling Development

by
Kaitlin A. Gerber

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APPROVED:

_____

Major Professor, representing Sustainable Forest Management

_____

Head of the Department of Forest Engineering, Resources, and Management

_____

Dean of the Graduate School

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Kaitlin A. Gerber, Author

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# CONTRIBUTION OF AUTHORS

R. Kasten Dumroese supplied resources including greenhouse space for the project focused on black locust and was instrumental in the design of this experiment. Anthony S. Davis assisted in designing the experiment and editing the manuscripts. Amy Ross-Davis assisted in preparing the manuscripts. Steven Perakis supplied materials needed for all nutrient analysis on koa.

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# Simulated environmental stressors influence Fabaceae seedling development

## Chapter 1: Introduction

The growth form and physiology of seedlings are controlled by genetic and environmental factors. Phenotypic plasticity makes it possible for seedlings to employ different compensatory mechanisms to tolerate environmental stressors (Chapin 1991). Seedling establishment and development is often influenced by a suite of environmental factors including temperature (Nobel and Zutta 2005), moisture (Pinto et al. 2016; Nobel and Zutta 2005), light interception (Farque et al. 2001), atmospheric $CO_2$ concentrations (Dang et al. 2008), soil fertility (Oliet et al. 2005), disturbance events (i.e. Crotteau et al. 2013), herbivory (Ameztegui and Coll 2015), and competition from other plants (Denslow et al. 2006; Parker et al. 2012). The importance of identifying traits associated with survival in low resource environments has long been recognized (Chapin 1991). Because it is rare that seedlings are planted on optimal sites, identifying ecophysiological responses to stressors can be advantageous in growing seedlings able to overcome constraints to survival. If nursery-grown seedlings develop in a way that builds from the species' adaptive phenotype, it is possible to cultivate seedlings with higher survival after encountering stresses that occur following planting.

By studying seedling morphology and physiology throughout the 20th century and refining those observations largely from 1990 onwards, it is known that certain characteristics associated with tolerance of stressors and can been used to predict post-planting seedling survival (Dumroese et al. 2016; Grossnickle 2012; Dumroese et al. 2005; Chapin 1991). Morphological attributes of seedlings are usually most easily measured and often include height,

root-collar-diameter, root to shoot dry biomass, and height to diameter (Dumroese et al. 2005).

However, it is important to include physiological tests when possible to more completely

describe the status of the seedling and because seedlings that appear to have a quality growth

form may not actually be of high physiological quality (Grossnickle 2012; Stone 1995; Stone and

Jenkins 1971). Physiological tests often include measures of photosynthesis, water potential, root

growth potential, and mineral nutrition (Dumroese et al. 2005). Each physiological test is useful

in describing different features of the seedling (i.e. ability to grow, tolerate drought, or egress

roots).

Seedling phenotype is partially site dependent; certain traits are advantageous under

different environmental stressors. For example, on sites where competition from other plants is

severe, taller seedlings capable of rapid growth are generally better suited for outgrowing

competing vegetation (Johnson and Smith 2005; Grossnickle 2012). Measuring instantaneous

variables, such as net photosynthetic assimilation, can provide insight on growth capacity and

help predict seedling survival when competing vegetation is limiting the establishment of planted

species. As well, on sites where moisture is limiting, seedlings that are slow-growing with

shorter shoots and sturdy root systems are often better suited to survive (Cregg 1994;

Grossnickle 2012). On sites where edaphic variables limit seedling growth and survival, root

growth potential may be a more appropriate physiological test to predict post-planting survival

(Ritchie and Dunlap 1980).

Of the morphological measurements employed to evaluate seedlings, root-collar-diameter

(RCD) is often described as the most reliable predictor of seedling survival, and seedlings with

larger RCD have been found to have greater survival than those with small RCD (Johnson and

Cline 1991; Grossnickle 2012; Mexal and Landis 1990). A seedling's RCD is positively

correlated with the size of the root system, and larger root systems with more surface area can uptake more water and nutrients, and can access water deeper in soil profiles to support physiological processes (Mexal and Landis 1990). The root to shoot dry biomass and height to diameter ratios of seedlings can describe drought avoidance capacity; well-balanced shoot and root systems are often associated with increased survival (Grossnickle 2012); there is little agreement, however, on what that balance or ratio should be across species and site conditions. When possible, assessing seedling physiology as it relates to the limiting environmental resource after morphological adaptations are assessed can help ensure seedlings are fit to survive and grow on the planting site (Dumroese et al. 2005).

Both species under examination, black locust (*Robinia pseudoacacia* L.) and koa (*Acacia koa* A. Gray), are valued in restoration projects in part for their positive impact on soil. Through forming symbiotic relationships with rhizobium or *Bradyrhizobium*, these species are able to fix atmospheric nitrogen (N). Soil N pools can successively increase, which is then available for use by other plant species (Scowcroft et al. 2004; Hong et al. 2013). Their large influence on the soil quality and high utility in restoration generates a demand for the assessment of seedling development under environmental stressors. Degraded sites are often more difficult to restore, thus, assessing ecophysiological traits associated with successful seedling establishment under environmental stressors will provide insight to the adaptive phenotype of the seedlings. The goal of this thesis is to assess compensatory strategies employed by koa in conditions of low water and phosphorus (P) and to assess the effect of rhizobium inoculation and fertilizer interaction on black locust seedlings in a way that can reduce pollution caused by fertilization without compromising seedling morphology.

Chapter 2 describes an experiment to identify how reduced water and P, two characteristics common to the native range of this species, impact koa seedling morphology, physiology, and mineral nutrition. This species-specific information, which describes the natural ecophysiological compensatory strategies under these limiting conditions, can then be used to guide target seedling attributes to specific site conditions. It is possible that seedlings can be conditioned for the stressors they will encounter upon outplanting by being subjected to those conditions in the nursery and may contain the traits needed for successful establishment. Through incorporating ecophysiological knowledge into the way seedlings are grown, seedling development can be better controlled to cultivate seedlings with characteristics linked to survival.

The experiment described in Chapter 3 seeks to determine the combination of fertilizer application rate and rhizobium inoculation status that produces the largest black locust seedlings and with the highest nodule formation. The goal of this experiment is to determine if less fertilizer can be used while achieving similar growth if seeds are inoculated with rhizobium before sowing. This would generate less fertilizer runoff without compromising the morphological specifications of the target black locust seedling.

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**Chapter 2: Evaluating the ecophysiological response of koa (*Acacia koa* A. Gray) seedlings to reduced water and phosphorus**

## Introduction

Interest in forest restoration using koa (*Acacia koa* A. Gray), a dominant overstory tree species endemic to Hawaii, has increased over the last several decades because this species provides important ecosystem services and potential economic returns through specialty wood products. Agricultural land use conversion largely driven by cattle ranching and sugarcane production during the 19th century prompted the clearing of native Hawaiian forests to such an extent that only approximately 10% of original koa forest cover remains (Cuddihy and Stone 1990; Wilkinson and Elevitch 2003). Subsequent factors including introduced ungulate grazing (Spatz and Mueller-Dombois 1973; Baker et al. 2009), competition from invasive plants (Denslow et al. 2006; Pinto et al. 2015), mortality induced by koa wilt disease (Dudley et al. 2017), and edaphic limitations (Scowcroft et al. 2007) have made it difficult for new forests to establish. This loss is of special concern to the persistence of the many bird species listed as federally endangered that occur within koa forests (Baker et al. 2009). As a native, fast growing, N-fixer, koa is able to quickly provide habitat to endangered species (Pejchar et al. 2005) while improving soil quality (Leary et al. 2004). The scarcity of koa forests coupled with rising interest in restoration has generated a demand for prompt and effective forest regeneration.

In Hawaii, limited water and phosphorus (P) have been shown to impact koa regeneration and productivity (Scowcroft et al. 2007; Ares and Fownes 1999; Cole et al. 1996). Phosphorus is commonly a scarce nutrient in highly weathered tropical soils (Dalling et al. 2016); Hawaii contains several orders of soil low in P. Andisols, the most prominent soil order in Hawaii, have

a high water holding capacity but are high in aluminum and iron, two minerals that adsorb P, making it less accessible to plants (Deenik and McClellan 2007; Hiradate and Uchida 2011). Other soil orders of Hawaii deficient in P include highly weathered and infertile Oxisols and Ultisols which have a strong capacity to adsorb P, and sandy, poorly developed Entisols (Deenik and McClellan 2007). Rainfall can similarly impact the amount of P in soil such that increased rainfall often leads to greater leaching and binding from cations (Austin and Vitousek 1998).

The rainfall patterns of Hawaii are highly variable and are a result of the east-northeast trade winds and land thermal effects caused by orographic lifting (Giambelluca et al. 2013). Across the Hawaiian Islands, windward mountain slopes typically have a higher mean annual rainfall whereas leeward lowlands or areas at the greatest elevations typically have a lower mean annual rainfall. Koa forests naturally occur over a wide gradient of precipitation, ranging from 850 to 5,000 mm of rainfall annually (Friday 2011). The amount of water available to koa seedlings has been found to have a profound impact on forest regeneration where growth is limited on drier sites (Harrington et al. 1995).

In environments where water or P availability is limited, seedlings can employ a variety of compensatory strategies that involve altering their morphology and physiology. In response to low water or P, other species have shifted carbon allocation from shoot to roots, thereby increasing their root to shoot ratio (R:S) (Kolb et al. 1990). Just as the R:S is important in supplying edaphic resources to the shoot, the architecture of the root system can influence its ability to capture resources. Plants grown in water-limited conditions typically adapt their root architecture by growing a longer taproot to capture water stored in deeper soil profiles (Ho et al. 2004; Alsina et al. 2011; Canadell et al. 1996). Conversely, P is found at shallow soil profiles,

which encourages greater lateral soil exploration by the root system (Fang et al. 2009; Lynch 1995).

Physiological measurements used to evaluate seedling quality include water-use efficiency (WUE), photosynthesis, pre-dawn water potential ($\Psi_{PD}$), and mineral nutrition (Dumroese et al. 2005). By increasing their WUE, plants can increase the ratio of carbon (C) gained to water lost through transpiration in photosynthesis (Ares et al. 2000). Thus, seedlings that exhibit a high WUE will be better able to accumulate biomass under conditions of low soil moisture. The instantaneous rate of $CO_2$ assimilation can be used to predict growth ability; seedlings of a low physiological quality have been found to exhibit poor growth rates (Lahamedi et al. 1996). Low soil moisture can cause complete or partial stomatal closure, creating a smaller pore space for C diffusion as a response to reduce water loss via transpiration, causing a decline in photosynthetic assimilation (Hetherington 1998). Other species of *Acacia* have been found to be highly tolerant of xylem cavitation (Maherali et al. 2004), possibly through closing stomata thereby relieving cavitation pressure (McDowell et al. 2008). Low P can similarly decrease photosynthetic assimilation because P is an essential element that makes up many of the molecules used in photosynthesis and respiration (Ellsworth et al. 2015).

Mineral nutrition can similarly be used to help predict seedling growth and survival. Seedlings that have high N concentrations have been found to display an increased ability towards rapid root egression and photosynthetic assimilation, which are essential in coupling to the planting site (Villar-Salvador et al. 2012; Oliet et al. 2013). High seedling P content was positively correlated with successful initial establishment for a different species of *Acacia* (Oliet et al. 2005). The mineral nutrition of seedlings can be manipulated through nursery and field fertilization.

In this study, we examined how koa seedlings grown for 17 weeks in a greenhouse adapted their growth form, physiology, and nutrient status under reduced water (W; 65W vs 85W) and P (0P vs 15P). It is hypothesized that: 1. Reducing the amount of W or P will cause a decline in growth and rate of $CO_2$ assimilation; 2. Seedlings will increase root length under reduced W, whereas under reduced P seedlings will develop wider root systems; 3. Seedlings under reduced W treatments will increase their WUE and will show slightly higher $\Psi_{PD}$ values resulting from water stress; 4. Nutrient concentrations will decline under reduced W and P treatments; and, 5. Reductions in both W and P will exceed the effects of each stressor alone. By understanding the effect of specific stressors of the planting site on seedling ecophysiology, it is possible that seedlings can be cultivated to better overcome limitations to survival.

## Materials and methods

### Nursery culture

Koa seeds sourced near Koke'e State Park on Kauai were supplied by Nicklos Dudley (Hawai'i Agriculture Research Center, Kailua, HI). Seeds were mechanically scarified using a nail clipper before being heat stratified in a pot of water held at 90°C for 2 minutes and left to soak overnight as described in Wilkinson and Elevitch (2004).

Sowing took place at Oregon State University's Oak Creek Greenhouse on 22 May 2017 in Corvallis, Oregon (44°33'N, 123°17'W). # 7-S Nursery Containers (Grower's Nursery Supply, Inc., Salem, OR) measuring 35.5 cm height x 28.9 cm diameter with 6 extra 1 cm holes drilled into the bottom were used. A ~3 cm layer of pumice was placed at the bottom of each pot to enhance drainage before a media containing a 1:1:1 ratio of Sphagnum peat moss, perlite, and pumice was used to fill the pots. The sown seeds were covered with Deluxe (0.5 OZ.) Crop

Protection & Over-Winterized™ germination fabric (DeWitt Company Inc., Sikeston, MO) and misted 2-3 times daily until germinated. At this time the germination fabric was cut into individual disks and placed over the top of each pot. Seedlings were grown for 17 weeks under modified W and P treatments before being measured and destructively harvested.

**Water and P treatments**

The nursery experiment consisted of a factorial structure: 2 water treatments (65W and 85W) × 2 P treatments (0P and 15P). Seedlings in the 85W treatment received enough water to saturate the pot only after declining past 85% gravimetric weight content (GWC) while those in the 65W treatment received water to bring the pot to 85% GWC after declining past 65% GWC (Dumroese et al. 2015). Seedlings in the 15P treatment received Jack's® Professional Geranium™ 15-15-15 NPK fertilizer (JR PETERS INC., Allentown, PA) while those in the 0P treatment were given fertilizer deficient in P (Peter's® Professional Peat-Lite® Dark Weather Feed™ 15-0-15 NPK fertilizer; JR PETERS INC., Allentown, PA).

A modified exponential fertilization regime delivered 100 mg of fertilizer to each seedling weeks 3-5, 405 mg on week 6, and 865 mg weeks 7-17. Each seedling received 10.2 g Total Applied Fertilizer. If the pot did not decline past the designated GWC (i.e. 65% or 85%) within the week, fertilizer solubilized in 500 mL of water was delivered to the seedling to ensure equal fertilization. Background P levels in the water system were 0.002 mg/L $PO_4$-P, which was considered insignificant. Although the micronutrients differed between the two fertilizers, levels were assumed to be within the required range to not impact growth (Table 2.1).

**Measurements**

Data collection began on 25 September 2017, after the final week of fertilization. Height and root-collar-diameter (RCD) were measured on each seedling.

The instantaneous rate of $CO_2$ assimilation ($A$), instantaneous WUE ($A/E$), and $\Psi_{PD}$ (Bars) were measured twice on each seedling to determine if seedlings were able to quickly recover from water stress. The first series of measurements were taken once the media declined past treatment water content (i.e. 65% or 85% GWC). After the first measurement was completed, water was delivered to return media to target GWC (i.e. 85% or 100%). Seedlings were left overnight to uptake water; the following day the second series of measurements were taken.

Instantaneous rate of $CO_2$ assimilation and instantaneous WUE measurements were taken between 11am-2pm using a LI-6400xt equipped with a broad leaf chamber (Li-COR, Inc., Lincoln, NE). Inputs used were as follows: 24°C block temperature, reference $CO_2$ concentration of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, relative humidity between 55-60%, and a PAR value of 1100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Instantaneous rates of $CO_2$ assimilation were logged once the chamber reached a 3/3 steady state. The measured bipinnately compound juvenile leaves selected from the upper half of the seedling were clipped and leaf area was calculated and used to correct the leaf area input (Schindelin et al. 2012). $\Psi_{PD}$ data was collected using a PMS pressure chamber (PMS Instruments, Albany, OR).

The roots of each seedling were separated from their shoot and carefully washed free of media in a water bath. Root structural features measured included length of longest root and root width. Root width was measured as the widest lignified point nearest the stem before tapering off. The number of nodules on each root structure was counted. Roots and shoots were dried in an oven at 60°C for 48 h before shoot dry mass (SDM) and root dry mass (RDM) measurements were collected.

A subset of 12 randomly chosen seedlings from each treatment was selected for foliage, stem, and root C, N, and P analysis. The foliage of the selected seedlings was stripped from the stem and placed in a separate labeled paper bag. Paper bags containing the plant organs were dried in an oven at 60°C for 48 h before dry biomass measurements were collected. Samples were ground and re-dried for 24 h before 2.3-2.7 mg of foliage, 2.6-3.0 mg of stem, and 3.0-3.4 mg of root were weighed out in 5 mm x 9 mm tin capsules. A Costech EC-4010 elemental combustion analyzer (Costech Analytical, Valencia, CA) was used to run the C and N analysis. Atropine was used as the standard, apple leaf served as the reference material, and approximately 5% of the samples were run in duplicate.

P analysis was carried out using acid digestion: 1.9-2.1 g of finely ground foliage, stem, or root was ashed in glass scintillation vials for 12 h at 475°C before 20 mL of 0.5N HCL was added to the vial once cool. Total P was measured using a Lachat Quickchem 800 colorimetric auto-analyzer (modified Method #10-115-01-1-0, instead using nanopure water) (Lachat Instruments, Milwaukee, WI). The same reference material and duplicate frequency were used as the C N analysis.

Extracellular phosphatase activity assays were conducted on the artificial growing media. Briefly, 1g of dry media was homogenized in 100 mL of 50 mM pH 5 sodium acetate buffer to create a media slurry. The sample and assay reagents were loaded into plates. Assay reagents included a substrate solution of 50 μl 4-methylumbelliferone-phosphate (4-MUB-phosphate), a standard solution of 50 μl 4-MUB, a media solution of 200 μl media slurry, and the phosphatase assay 200 μl media slurry and 50 μl substrate solution; the substrate and standard solution contained 200 μl of sodium acetate buffer while the soil solution contained 50 μl of sodium acetate buffer. Each sample contained three quench controls made of 50 μl standard solution and

50 μl substrate solution. Plates were covered in aluminum foil and incubated at room temperature for 4 h, 10 μl of 0.5N NaOH was added to each well to terminate incubation. A Synergy™ 2 microplate reader set with an excitation wavelength of 360/40 nm and temperature of 26 °C was used to measure florescence (BioTek[®] Instruments Inc., Winooski, VT) before activity (nmol g$^{-1}$ h$^{-1}$) was calculated (German et al. 2011).

**Design and analysis**

We used a completely randomized design consisting of 22 seedlings per treatment. Each seedling was treated as a single replication. Once germinated, seedlings were randomly assigned to treatment groups the first day of week 3. Initial height was not significantly different among the treatments ($P = 0.869$). An extended factorial ANOVA was used to compare the main effects of reduced W and reduced P and their interaction (reduced W*P) on the measured variables with significance defined as $P < 0.05$. Tukey's pairwise comparison was subsequently used when main effects interacted. A negative binomial generalized linear model was used to analyze nodule formation. A Wilcoxon signed rank test was used to compare seedling physiology (i.e. rate of $CO_2$ assimilation, WUE, and $\Psi_{PD}$) before and after growing media were returned to target GWC. When examined individually, before data were log transformed. Data were analyzed using R version 3.4.0 software (R Foundation for Statistical Computing, Vienna, Austria).

## Results

**Morphological differences in height, RCD, SDM, RDM, and R:S**

Exposure to reduced W or P significantly impacted all morphological characteristics measured (Table 2.2). Seedling height was significantly lower only when treated with a combination of

reduced W and P (i.e., 65W and 0P), which also resulted in significantly higher R:S compared to all other treatment combinations (Table 2.2; Figure 2.1). Seedlings treated with 65W had 26% smaller RCD, 39% less SDM, and 16% less RDM compared to seedlings treated with 85W, irrespective of P treatment (Table 2.2; Figure 2.1). Similarly, seedlings treated with 0P had 18% smaller RCD, 28% less SDM, and 26% less RDM compared with seedlings treated with 15P, irrespective of W treatment (Table 2.2; Figure 2.1).

**Root structural, nodule formation, and extracellular phosphatase activity**

By the end of the 17-week growing period, the roots filled the space of the pots. W significantly impacted root width, longest root length, and nodule formation such that seedlings treated with 85W produced 199% more nodules, a root width 34% larger, and a longest root length 8% greater relative to seedlings treated with 65W (Figure 2.2). Similarly, P significantly impacted nodule formation and root width such that relative to seedlings treated with 0P, those treated with 15P formed 146% more nodules and a root width 19% larger (Figure 2.2). Longest root length was unaffected by P treatments (53.5 ± 1.2 cm 0P vs. 55.1 ± 1.6 cm 15P). Neither W (493.5 ± 27.3 65W vs. 478.7 ± 31.0 85W) nor P (512.1 ± 27.5 0P vs. 458.1 ± 30.9 15P) significantly affected extracellular phosphatase activity (Table 2.2).

**Physiological differences in rate of $CO_2$ assimilation, WUE, and $\Psi_{PD}$**

Neither rate of $CO_2$ assimilation nor WUE differed before and after watering for seedlings in either treatment; however, relative to those treated with 15P, seedlings treated with 0P showed a significant decline in $\Psi_{PD}$ after watering (4.67 ± 0.37 vs. 3.80 ± 0.51; Table 2.5; Figure 2.3).

When before and after measures were examined separately, the rate of $CO_2$ assimilation, WUE, and $\Psi_{PD}$ differed between treatments (Table 2.3). Since the rate of $CO_2$ assimilation and WUE did not differ significantly before versus after watering, only after watering values are presented for simplicity. W significantly impacted the rate of $CO_2$ assimilation, WUE, and $\Psi_{PD}$ after watering (Table 2.3). Relative to seedlings treated with 85W, those subjected to 65W had a 61% lower instantaneous rate of $CO_2$ assimilation rate, 155% greater instantaneous WUE, and 46% greater $\Psi_{PD}$ (Figure 2.3). Neither the rate of $CO_2$ assimilation (15.6 ± 1.4 0P vs. 14.1 ± 1.3 15P) nor WUE (1.00 ± 0.31 0P vs. 0.8 ± 0.13 15P) differed between P treatments; however, seedlings treated with 0P had a 24% higher $\Psi_{PD}$ before watering relative to those treated with 15P (Table 2.3; Figure 2.3).

**Foliar and root C, N, and P concentrations and whole-plant C, N, and P content**

*Nitrogen*

Foliar N concentration was greater among seedlings treated with 65W and 15P compared to all other treatment combinations (Figure 2.4). Root N concentration was 10% higher among seedlings treated with 65W compared to 85W; P treatment did not affect root N content (2.03 ± 0.05 mg 0P vs. 1.96 ± 0.05 mg 15P). Whole-plant N content was 16% higher among seedlings treated with 85W compared to 65W and 25% higher among those treated with 15P compared to 0P (Table 2.4; Figure 2.4).

*Carbon*

Similarly, whole-plant C content was 30% higher among seedlings treated with 85W compared to 65W and 20% higher among those treated with 15P compared to 0P (Table 2.4; Figure 2.4).

Root C concentration was 2% greater among seedlings treated with 0P compared to those treated with 15P. W treatments did not affect root C concentration (45.93 ± 0.29 g 65W vs. 45.40 ± 0.29 g 85W). Foliar C concentration was 0.3% greater among seedlings treated with 85W compared to 65W (Figure 2.4). P treatments did not significantly affect foliar C concentration (50.61 ± 0.83 0P vs. 50.15 ± 0.16 g 15P)

*Phosphorus*

Whole-plant P content was greatest among seedlings treated with 15P at 85W, followed by 15P at 65W, 0P at 85W, and 0P at 65W (Figure 2.5). A similar trend was observed for root P concentration, with the exception of no significant difference between 65W and 85W at 0P (Table 2.4; Figure 2.5). Foliar P concentration was 22% higher among seedlings treated with 85W compared to 65W and 231% higher among those treated with 15P compared to 0P (Figure 2.5).

## Discussion

The hypothesis that reducing the amount of W or P available to koa seedlings will cause a decline in growth and rate of $CO_2$ assimilation was partially confirmed. Seedlings grown under reduced W or P treatments accumulated significantly less biomass with smaller RCD. Unlike those grown under reduced W treatments, which had a reduced $CO_2$ assimilation rate, seedlings treated with reduced P did not differ in rate of $CO_2$ assimilation. Other species of tree seedlings under drought stress have similarly displayed reduced photosynthetic assimilation and biomass accumulation (Lamhamedi et al. 1996). These two parameters are predictably linked; seedlings

of low physiological quality exhibit poor growth rates compared to those of higher physiological quality (Mexal and Landis 1990; Lamhamedi et al. 1996).

Slow growth is a generalized compensatory strategy controlled by hormonal signaling to low resources because it reduces the reliance on the environment for new resources (Chapin 1991; Chapin et al. 1993). Rapid growth decreases stress resistance as a result of the increases to tissue turnover, greater losses of C, nutrients, and water, and decreased allocation to storage and plant defenses (Chapin et al. 1993). Slow rates of growth and photosynthesis are common to species adapted to low resource environments (Chapin 1991). The conservative biomass accumulation of seedlings treated with reduced W or P and its interactive effect on height provides insight on morphological adaptations of koa seedlings to limited W and P resources. Shorter seedlings have been found to perform better on droughty sites (Mexal and Landis 1990; Cregg 1994; McTague and Tiius 1996; Tuttle et al. 1987; Thompson 1986). Shorter seedlings typically have less foliage, making it easier for root systems to supply the water required to maintain a proper water balance (Grossnickle 2005). Though when only treated with reduced W or P seedling height did not differ, the interactive effect of W and P on shoot height indicates that on sites where both resources are both low in abundance, shorter seedlings may be better suited to site conditions.

Although smaller than seedlings receiving 15P, those grown under the 0P treatment did not differ in rate of $CO_2$ assimilation. Koa trees at different stages of development have been found to increase growth when fertilized with P (Earnshaw et al. 2016; Scowcroft and Silva 2005). In another study, koa seedlings displayed mixed responses to field P fertilization in terms of height, basal stem diameter, and biomass production; these parameters were all ultimately dependent upon the seed source (Scowcroft and Silva 2005). The seeds used in this study were

sourced from the Kokee region of Kauai, which has soils low in P (Pearson and Vitousek 2002). Consequently, it is possible that the mother tree was genetically adapted to low P and could photosynthesize without impairment under low P. Other studies have found the growth rate of koa to be controlled by genetic factors, specifically local seed sources achieved greater growth than trees from off-site seed sources (Scowcroft and Silva 2005). A number of metabolic changes to compensate for low P have been reported including the ability to operate with low ribosomal RNA and proteins (Sulpice et al. 2014), and the ability to use galactolipids and sulfolipids in place of phospholipids (Lambers et al. 2012), but no species-specific information is available on koa.

RCD is often described as the most reliable morphological predictor of survival measured on seedlings because it non-destructively predicts the size of the root system (McTague and Tiius 1996; Chavasse 1977; Grossnickle 2012). Seedlings with greater RCD typically have larger root systems with more exposed surface area and are better able to capture water to overcome drought stress (Thompson 1985; Grossnickle 2012; Grossnickle 2005). Even though seedlings exposed to 65W or 0P treatments did not develop a greater RCD or RDM relative to those treated with 85W or 15P, seedlings treated with 65W at 0P developed greater R:S. Exposure to limited W and P interacted to cause seedlings to shift resource allocation from shoot to root. This indicates that the formation of a sturdy root system at the expense of shoot growth is a compensatory strategy applied by koa under reduced water and P.

The R:S can be controlled when growing seedlings in an artificial setting. Container size and shape can influence the amount of root mass where larger containers produce more massive root structures (Pinto et al. 2015; Aghai et al. 2014). If growing seedlings in containers, it is important to coordinate the nursery-growing schedule to the outplanting date so that seedlings

are not overgrown when planted. Not only can that lead to the formation of seedlings that are tall and spindly with root structures unable to support water transport, but also it can make the roots bound to the pot unable to egress once planted. Selecting an appropriate container then setting acceptable height and RCD minima and maxima can be a valuable strategy in ensuring the planted seedlings have a well-balanced R:S (Grossnickle 2012; Dumroese et al. 2005).

The hypothesis that seedlings will increase their longest root length under reduced W whereas under reduced P seedlings will develop wider root systems was not confirmed. The pots used in this experiment were not large enough to avoid constricting the roots to the shape of the pot. Using large pots to sow seedlings increases the risk of mortality because the media can become waterlogged since small seedlings in the early stages of growth are not able to uptake large amount of water for transpiration. The waterlogged media can facilitate fungal growth, causing the roots to mold. All treatments were affected equally by the pot constriction. W affected root length in that seedlings grown under 65W developed a slightly shorter root system that those brought to saturation after declining past 85% GWC, however these results were confounded by the pot and are not biologically important. Root width was not affected by pot constriction and seedlings under reduced W or P treatments produced significantly narrower root systems relative to their respective controls (i.e., 85W or 0P).

It was hypothesized that seedlings under reduced W treatments will increase their WUE and will show slightly higher $\Psi_{PD}$ values resulting from water stress. This hypothesis was supported; seedlings treated with 65W displayed higher WUE and $\Psi_{PD}$. Seedlings under the reduced W treatment appear to have closed their stomata to reduce water loss, which is reflected by the lower rates of $CO_2$ assimilation and greater WUE. Genetic (Ares et al. 2000) and environmental (Ares and Fownes 1999) factors have been found to interact to determine the

WUE of koa. Locally adapted populations have been found to be more physiologically able to overcome constraints related to low soil moisture (Ares et al. 2000). Resistance to water stress and increased WUE can be partially controlled through seed selection and water stressing the seedlings in an artificial setting before planting. Providing seedlings with an abundance of water will not physiologically condition them for low water availability once outplanted (Abrams 1988; Grossnickle 2012). Failure to harden seedlings in the nursery to the anticipated water stress at the outplanting site will not trigger the physiological cues that a seedling can employ to develop protection from the stressor (Grossnickle 2012). Selecting an appropriate seed source then water hardening the seedlings should create more resilient seedlings that are physiologically conditioned to tolerate low water availability. Because water is a limiting factor to growth and productivity of koa, especially at higher elevations (Harrington et al. 1995; Ares et al. 2000; Ares and Fownes 1999), physiologically conditioning seedlings is thought to improve survival.

Seedlings treated with 0P recovered better from water stress after watering, displaying a mean $\Psi_{PD}$ value 22% lower than before watering. P has been shown to have a mitigating effect on drought stress for other species (Kuwahara et al. 2016; Lui et al. 2017; Tariq et al. 2017). This may explain why seedlings grown with 15P did not display a higher $\Psi_{PD}$; they could be better at avoiding drought stress since they were not physiologically impaired. Other studies on koa found that WUE responses were caused by stomatal rather than nutrient limitations to photosynthesis (Ares and Fownes 1999; Harrington et al. 1995), and the results of this experiment concur with these previous findings.

Despite having significantly more nodules than all other seedlings, those grown under control conditions (i.e., 85W and 15P) did not have a higher foliar or root N concentration. Seedlings treated with 85W had a significantly higher N content, a result of the larger seedling

biomass rather than additional N provided by N-fixation. Dumroese et al. (2009) found that inoculated koa seedlings had a mean root N concentration 8% greater than uninoculated seedlings, which developed fewer nodules. The authors described the pink internal color of the nodules as a means to quickly assess whether the nodules are actively fixing N. Nodules observed in this study were creamy white and the mean nodule formation in this study was nearly half of that reported by Dumroese et al. (2009). The relatively low amount of nodules that formed, which were presumably inactive as evidenced by color, is possibly why nodule formation did not affect N concentration.

The hypothesis that nutrient concentrations will decline under reduced W or P treatments was partially confirmed. Seedlings under 0P treatments did have lower nutrient concentrations and contents relative to those receiving 15P, however seedlings treated with 65W contained lower foliar and root P concentrations but higher foliar and root N concentration relative 85W seedlings. Most foliar N is associated with the photosynthetic enzyme RuBisCo (Lambers et al. 2008; Millard et al. 2007). Other studies have similarly found drought stress to impact P uptake more strongly than N and suggest that the effects are likely short term and will disappear once water becomes available (He and Dijkstra 2014).

Mineral nutrition is important in describing the seedling's capacity for root egression once outplanted. One study in a semiarid Mediterranean region, where soil moisture and nutrient availability are poor, found P fertility to be positively related to survival and RDM (Oliet et al. 2005). Increasing P delivery in fertilization may be important when seedlings are water-stressed in the nursery. N nutrition has been found to have an antagonistic effect on drought tolerance for other species (Villa-Salvador et al. 2013). More research to assess N and P fertilization rates and

seedling survival under conditions of low soil moisture could identify an appropriate fertilizer regime that balances nutrient loading and water stress.

## Conclusions

Cultivating nursery-grown seedlings with physiological and morphological attributes correlated with survival can improve outplanting performance (Grossnickle 2012), forming the premise of the Target Plant Concept (Dumroese 2016). This conceptual model brings forward that site-specific factors with the potential to limit outplanting survival should be anticipated and used to guide decisions around nursery production practices, serving to better connect field needs with nursery capacity (Dumroese et al. 2005). An understanding of how seedlings develop under conditions where resources are limited can be used to tailor the growing regime to produce high quality seedlings, whereby a high quality seedling is defined by its ability to survive and grow once outplanted (Duryea 1984; Landis and Dumroese 2006). Selecting an appropriate seed source, then cultivating seedlings to meet morphological specifications and conditioning seedlings to tolerate the stressor of the outplanting site should improve outplanting survival.

Seedlings in this study altered their physiological condition in response to conditions of low W or P. Seedlings subjected to reduced W or reduced P were smaller with reduced RCD, and had lower C, N, and P contents. Seedlings exposed to reduced W exhibited a suppressed ability to photosynthesize, but increased their instantaneous WUE as a compensatory strategy. Seedlings under 0P had a similar rate of $CO_2$ assimilation relative to those grown with 15P, suggesting that koa is able to employ strategies to avoid physiological impairment from conditions of limited P. Seedlings exposed to reduced W and P increased their R:S and were stunted in height. Future research is needed to assess whether these physiologically conditioned

seedlings will have greater growth and survival rates once outplanted onto these W and P limited sites. Results indicate on sites where plant-available water or P is limited, seedlings will accumulate less biomass. To cue physiological responses to overcome limited W, water-stressing seedlings in an artificial environment before planting on sites low in soil moisture may increase survival. Similarly, field P fertilization may enable seedlings to grow more rapidly, especially on sites where plant-available water is also limited.

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# Tables

**Table 2.1.** Specifications of the two fertilizers used. Seedlings subject to 15P treatments received Jack's® Professional Geranium™ 15-15-15 NPK fertilizer whereas seedlings subject to 0P treatments received Peter's® Professional Peat-Lite® Dark Weather Feed™ 15-0-15 NPK fertilizer

| Fertilizer Brand | Nitrogen | Available phosphate ($P_2O_5$) | Soluble potash | Water soluble Magnesium | Chelated Copper | Chelated Iron | Manganese | Molybdenum | Chelated Zinc | Boron | Calcium |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Jack's® Professional Geranium™ 15-15-15 NPK fertilizer | 2.88% ammoniacal 8.40% nitrate 3.72% urea  15% total N | 15% | 15% | 0.0500% | 0.0036% | 0.0500% | 0.0250% | 0.0009% | 0.0025% | 0% | 0% |
| Peter's® Professional Peat-Lite® Dark Weather Feed™ 15-0-15 NPK fertilizer | 2.0% ammoniacal 13.0% nitrate  15% total N | 0% | 15% | 2.0% | 0.0187% | 0.075% | 0.0375% | 0.0075% | 0.0375% | 0.0187% | 5% |

**Table 2.2.** Two-way ANOVA of the effect of W and P treatments on morphological and root structural variables of *Acacia koa* seedlings. Height, RCD, SDM, RDM, longest root, and root width are presented with F statistics with associated P-value whereas nodules formed is a presented with Z statistic with associated P-value.

| Variable | W | | P | | W*P | |
|---|---|---|---|---|---|---|
| | Test statistic | P-value | Test statistic | P-value | Test statistic | P-value |
| Height | 17.465 | 0.0001 | 16.543 | 0.0001 | 5.853 | 0.0177 |
| RCD | 40.321 | <.0001 | 32.46 | <.0001 | 0.458 | 0.5003 |
| SDM | 41.7874 | <.0001 | 38.4035 | <.0001 | 2.9517 | 0.0895 |
| RDM | 8.0295 | 0.0058 | 15.9363 | 0.0001 | 0.0005 | 0.9815 |
| R:S | 15.4071 | 0.0002 | 0.3794 | 0.5396 | 8.3453 | 0.0049 |
| Longest root | 4.657 | 0.0338 | 0.607 | 0.438 | 0.032 | 0.858 |
| Root width | 30.3275 | <.0001 | 10.2055 | 0.002 | 0.0596 | 0.8078 |
| Nodules formed | -3.081 | 0.00206 | -2.586 | 0.00972 | 1.094 | 0.27389 |

**Table 2.3.** Two-way ANOVA of the effect of W and P treatments on physiological variables of *Acacia koa* seedlings. (B) refers to before watering, (A) refers to after watering

| Variable | W | | P | | W*P | |
|---|---|---|---|---|---|---|
| | F statistic | P-value | F statistic | P-value | F statistic | P-value |
| Rate of $CO_2$ assimilation (B) | 49.7226 | <.0001 | 2.2754 | 0.1354 | 0.8686 | 0.3541 |
| Rate of $CO_2$ assimilation (A) | 23.05032 | <.0001 | 1.15375 | 0.2858 | 2.49872 | 0.1177 |
| Intrinsic WUE (B) | 0.43696 | 0.5105 | 0.9431 | 0.3344 | 0.85504 | 0.3579 |
| Intrinsic WUE (A) | 7.4327 | 0.0078 | 0.65655 | 0.4201 | 0.6348 | 0.4278 |
| $\Psi_{PD}$ (B) | 2.0716 | 0.1538 | 4.5871 | 0.0351 | 4.5871 | 0.1203 |
| $\Psi_{PD}$ (A) | 5.4324 | 0.0222 | 3.177 | 0.0783 | 3.368 | 0.07 |

**Table 2.4.** Two-way ANOVA of the effect of W and P treatments on nutritional status of *Acacia koa* seedlings

| Variable | W | | P | | W*P | |
|---|---|---|---|---|---|---|
| | F statistic | P-value | F statistic | P-value | F statistic | P-value |
| Foliar %C | 5.53 | 0.0233 | 3.51 | 0.0676 | 1.5 | 0.2265 |
| Foliar %N | 4.517 | 0.0392 | 18.364 | 0.0001 | 10.234 | 0.0026 |
| Foliar %P | 19.1376 | 0.0001 | 124.8748 | <.0001 | 1.1901 | 0.2813 |
| Root %C | 0 | 0.8305 | 16 | 0.0002 | 1 | 0.3144 |
| Root %N | 7.566 | 0.0086 | 0.562 | 0.4575 | 0.274 | 0.6031 |
| Root %P | 0.0662 | 0.7982 | 974.9335 | <.0001 | 26.4755 | <.0001 |
| Whole-plant C content | 16.3557 | 0.0002 | 22.0619 | <.0001 | 0.0502 | 0.8237 |
| Whole-plant N content | 4.9164 | 0.0318 | 35.1197 | <.0001 | 1.3861 | 0.2454 |
| Whole-plant P content | 19.6230 | 0.0001 | 186.8962 | <.0001 | 8.3972 | 0.0058 |
| Phosphatase activity | 0.4014 | 0.5288 | 1.7505 | 0.1908 | 0.0064 | 0.9366 |

**Table 2.5.** Wilcoxon signed-rank of before and after watering comparisons of physiological characteristics treated with reduced W or P

| Variable with treatment | W value | P-value |
|---|---|---|
| Rate of $CO_2$ assimilation | | |
| W | 234 | 0.7436 |
| P | 231 | 0.8034 |
| WUE | | |
| W | 277 | 0.1587 |
| P | 243 | 0.5798 |
| $\Psi_{PD}$ | | |
| W | 174.5 | 0.1156 |
| P | 79.5 | 0.0001416 |

# Figures
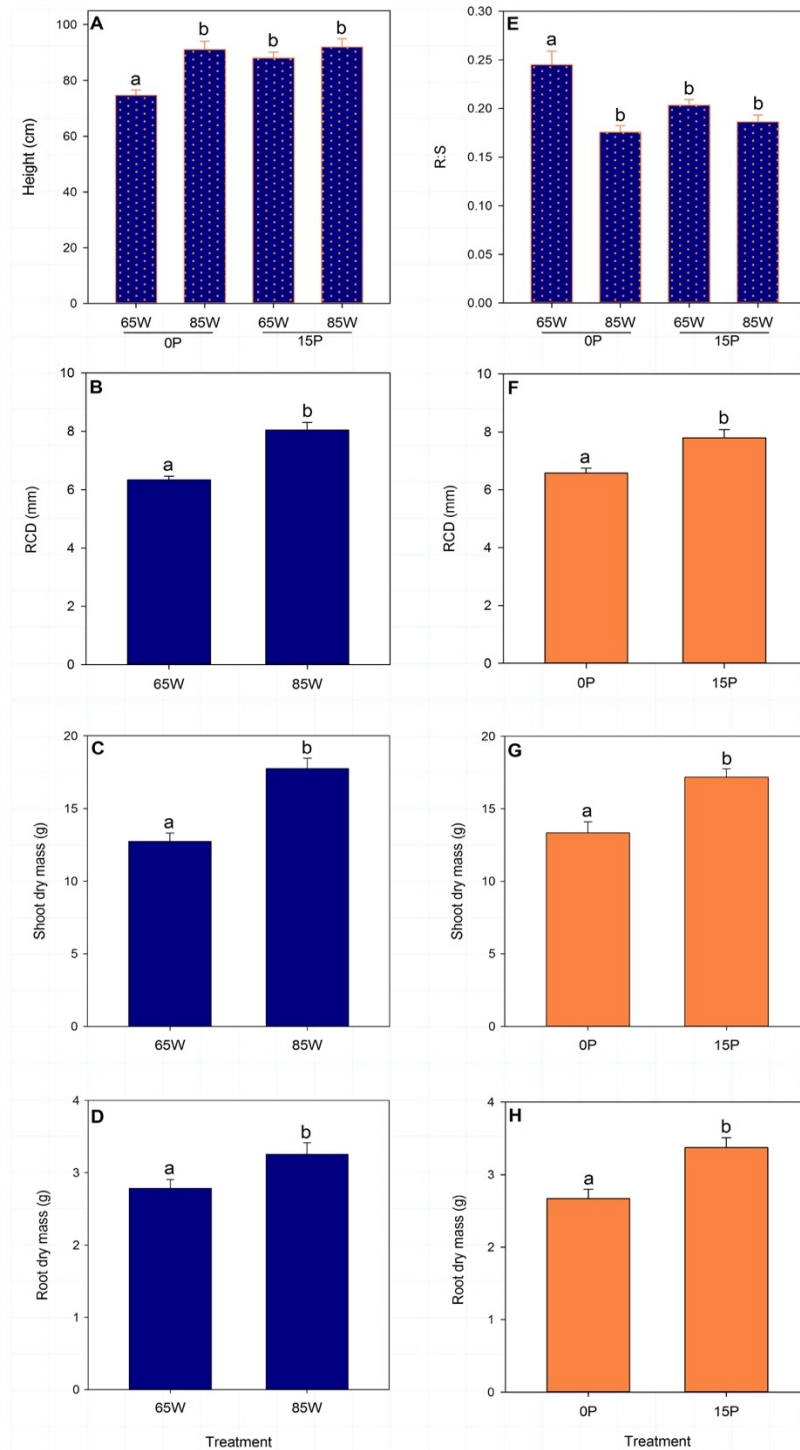


**Figure 2.1.** Means (± SE) of *Acacia koa* seedling (A) height (cm), (B and F) RCD (mm), (C and G) shoot dry mass (g), (D and H) root dry mass (g), and (E) R:S under different W (blue) and P treatments (orange bars). Different letters indicate significant differences (α = 0.05), interactions between W and P were included when significant
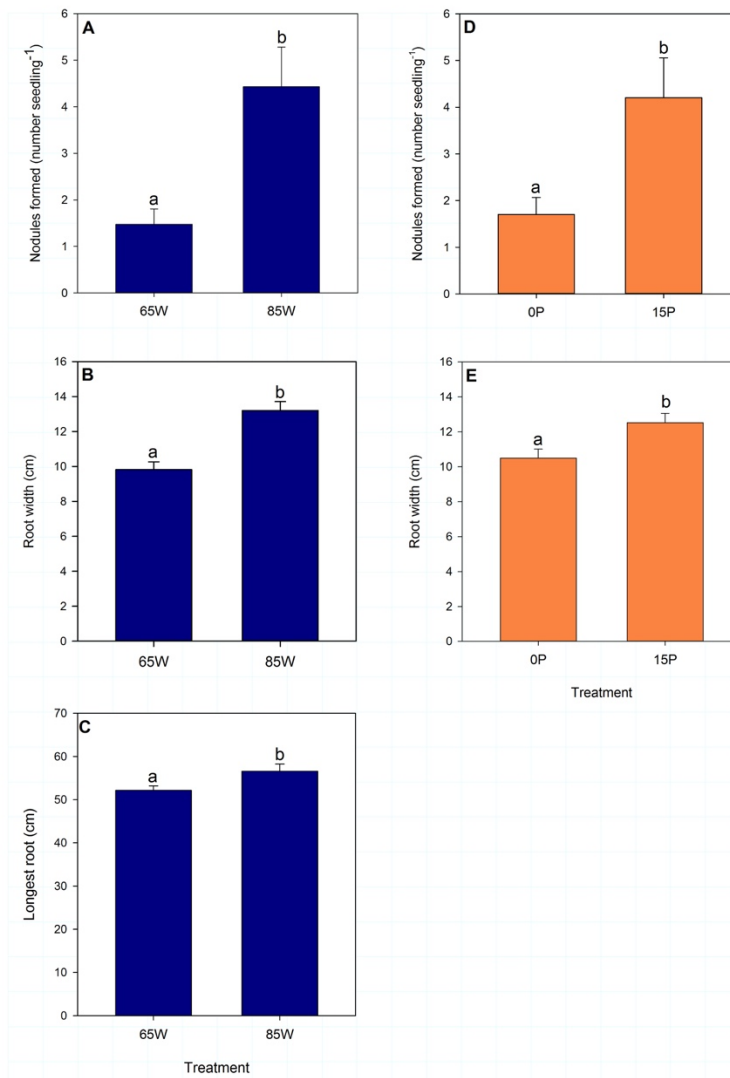
**Figure 2.2.** Means (± SE) of *Acacia koa* seedling (A and D) nodules formed (number·seedling$^{-1}$), (B and E) root width (cm), and (C) longest root (cm) under different W (blue) and P treatments (orange bars). Different letters indicate significant differences ($\alpha = 0.05$), interactions between W and P were included when significant
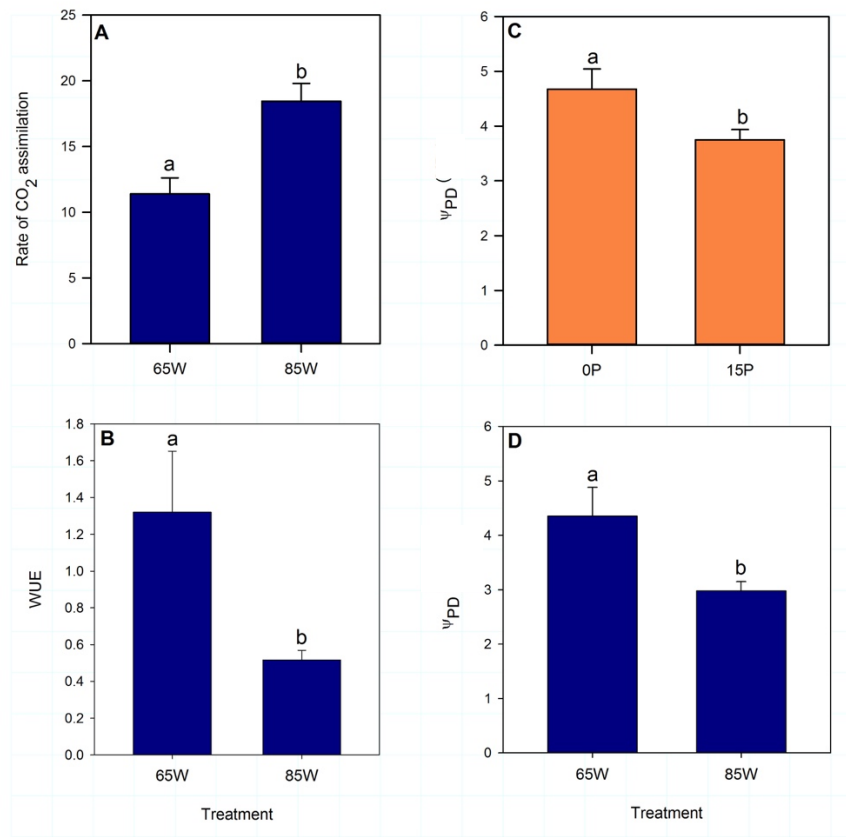
**Figure 2.3.** Means (± SE) of *Acacia koa* seedling (A) rate of CO2 assimilation after watering, (B) WUE after watering, (C) $\Psi_{PD}$ (Bars) before watering, (D) $\Psi_{PD}$ (Bars) after watering under different W (blue) and P (orange bars) treatments. Different letters indicate significant differences ($\alpha = 0.05$), interactions between W and P were included when significant
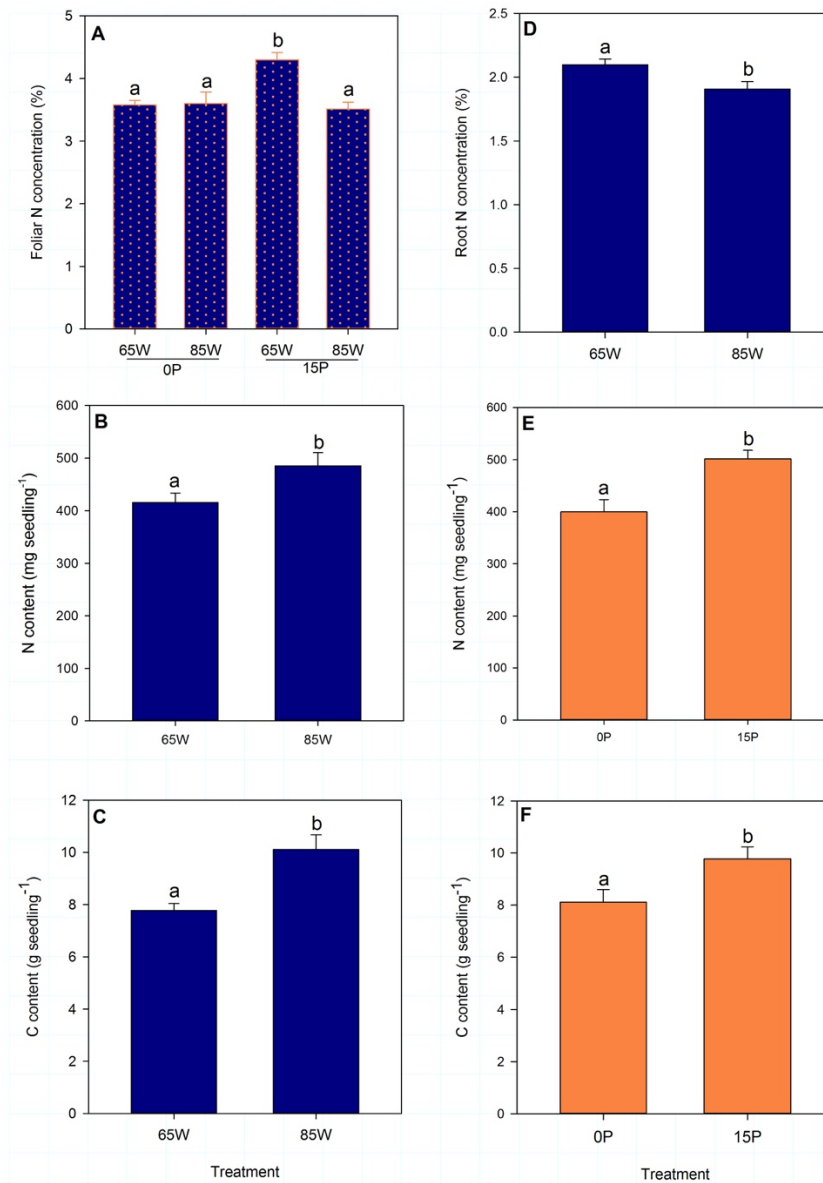
**Figure 2.4.** Means (± SE) of *Acacia koa* seedling (A) foliar N concentration (%), (B and E) whole-plant N content (mg·seedling$^{-1}$), (C and F) whole-plant C content (g·seedling$^{-1}$), and (D) root N concentration (%) under different W (blue) and P (orange bars) treatments. Different letters indicate significant differences (α = 0.05) interactions between W and P were included when significant

**Figure 2.5.** Means (± SE) of *Acacia koa* seedling (A and C) foliar P concentration (%), (B) root P concentration (%), and (D) whole-plant P content (mg·seedling$^{-1}$) under different W (blue) and P (orange bars) treatments. Different letters indicate significant differences (α = 0.05), interactions between W and P were included when significant
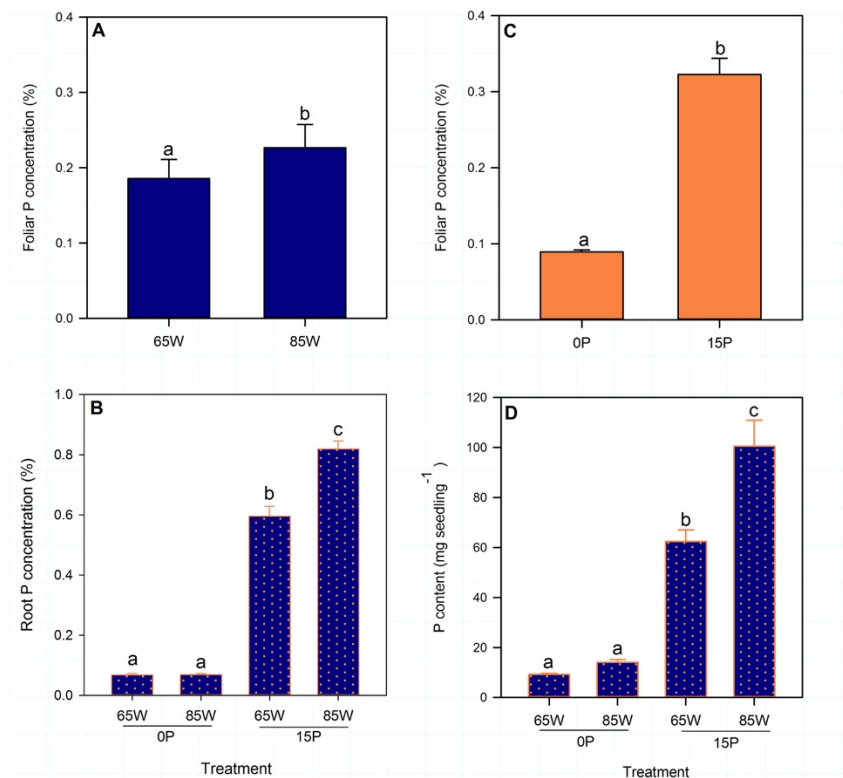
**Chapter 3: The interaction between fertilizer rate and rhizobium inoculation on black locust (*Robinia pseudoacacia* L.) seedling growth**

## Introduction

Black locust (*Robinia pseudoacacia* L., Fabaceae) is a N-fixing tree species native to the Ozark and Appalachian Mountain regions of North America. This species is valued in restoration worldwide for its capacity to improve soil quality and rapidly grow in conditions other species find unsuitable (Grünewald et al. 2009; Papaioannou et al. 2016; Yüsek 2012; Vítková et al. 2015; Buzhdygan et al. 2016; Qiu et al. 2010). In post-mining landscapes, black locust can be planted to improve soil properties and later harvested for biomass production (Grünewald et al. 2009). Soil nutrient depletion associated with short rotation forestry typical in biomass production can be mitigated using N-fixing species (Roberts et al. 1983). Soil quality is quickly improved after black locust is established on a landscape through increasing soil organic matter, total N, hydraulic conductivity, and water holding capacity (Papaioannou et al. 2016; Yüsek 2012). Despite the wide-ranging use of this species in restoration, little research has been conducted to assess the fertilizer rate and rhizobium interaction that produces the largest container grown seedlings with the high nodule formation while minimizing the production of fertilizer wastewater.

Determining the appropriate fertilizer application rate in container nursery operations is essential in cultivating seedlings that exhibit desirable growth rates (Grossnickle 2012; Jackson et al. 2012; Dumroese et al. 2011; Chaukiyal et al. 2013) and are best conditioned to survive once outplanted (Henderson et al. 1994).

Seedlings grown in nutrient deficient conditions are often stunted in height (Oliet et al. 2005; Dumroese et al. 2009; Dumroese et al. 2011). Shoot height has been positively correlated to growth capacity once outplanted; larger seedlings typically have more foliage and are able to photosynthesize at greater rates (Grossnickle 2012; Armson and Sadreika 1979; Mexal and Landis 1990). However, providing seedlings with excessive fertilizer can cause an increase in local surface and groundwater pollution form nursery runoff (Cregg et al. 2004; Juntunen et al. 2002). Other studies reported up to 60% of applied N (Dumroese et al. 1995) and up to 64% of applied P are discharged from the nursery (Juntunen et al. 2002). Identifying ways to reduce fertilizer use while maintaining seedling quality is important in mitigating nursery waste and pollution.

Species able to form symbiotic relationships with rhizobium may be able to achieve comparable growth under reduced fertilizer rates if inoculated (Yadav and Verma 2014). Once bacteriods are established within the root cells of the host plant, rhizobium can convert atmospheric N to ammonia (Sprent and McKey 1994), giving plants a competitive advantage in low N environments. Rhizobia are not found in the sterile growing media used in container nurseries; inoculating seeds with complimentary strains of rhizobia is essential for the formation of nodules and subsequent N fixation (Dumroese et al. 2009). Studies examining other leguminous species have found inoculation to increase seedling biomass and nodule formation (Chaudhary et al. 2011; Yadav and Verma 2014; Sánchez et al. 2014; Chaukiyal et al. 2013; Dumroese et al. 2009).

The objective of this study is to determine the combination of weekly fertilizer

application rate and rhizobium inoculation that produces the largest black locust

seedlings with high nodule formation while minimizing fertilizer usage. If too little

fertilizer is delivered to a seedling, growth can be reduced (Carfagna et al. 2011).

Conversely, under conditions of excessive nitrogen fertilizer, nitrogenase activity

within the bacteroids can be inhibited (Luciñski et al. 2002), reducing the amount of

N fixed by rhizobium. It is hypothesized that under a medium fertilizer rate (2 mg

Applied Fertilizer·seedling$^{-1}$·week$^{-1}$), seedlings inoculated with rhizobium will

produce the greatest growth and nodule formation.


## Materials and methods

### Nursery culture and experimental design

Black locust seedlings were grown in a greenhouse at the USFS Rocky Mountain

Research Station in Moscow, Idaho, USA (46.7232, -117.0029). Seeds were obtained

from a Kentucky (USA) source (collected in 2012 and distributed by Lawyer Nursery

Inc., Plains, Montana, USA). Hot water scarification was used to break seed

dormancy on 6 September 2016 before being sown into Ray Leach Cone-tainer™

supercells (164 cm$^3$, 3.8-cm diameter, 21-cm depth; Stuewe & Sons, Inc., Tangent,

OR, USA) such that each treatment contained 10 seedlings. A growing media

comprised of 40%-50% Canadian Sphagnum peat moss, vermiculite, and fine-aged

bark (Sunshine Professional Custom Mix, SunGro® Horticulture, Agawam, MA,

USA) was used. Seeds in the inoculated treatments were coated by hydrated black

locust specific rhizobium through gentle mixing (Plant Probiotics Company,

Indianapolis, Indiana, USA). The inoculated treatments were sown last to avoid cross-

contamination to uninoculated seeds. A layer of forestry grit was used to cover the

seeds once sown into the containers which were misted with water 3 times daily until

seeds germinated (Target® Forestry Nursery Grit, Burnaby, BC, Canada). Weekly

fertigation began 2 weeks after seeds were sown. Wil-Sol® Pro-Grower 20-7-19 NPK

(Wilber-Ellis Company, Aurora, CO, USA) was alternated with YaraLiva®

CALCINIT™ 15.5-0-0 NPK (Yara North American, INC, Tampa, FL, USA) which

ensured seedlings received all necessary nutrients at the target rates of 0, 2, or 4 mg

Applied Fertilizer·seedling$^{-1}$·week$^{-1}$. Seedlings were fertigated with the designated

fertilizer rate once per week as the container declined past 85% gravimetric weight

content (GWC). Thus, the study design was a completely randomized design with two

factors (3 fertilizer rates x 2 inoculation treatments). The six treatments were

randomly rearranged following each fertigation event to minimize greenhouse effects.

**Growth measurements**

Seedling height and caliper were measured on the first day of fertigation to

ensure no morphological differences existed prior to beginning the experiment. Final

measurements were collected 10 weeks after the seeds were sown. Height and RCD

were measured; relative height growth and relative caliper growth were calculated as

the difference between the final and initial values. Roots were washed clean of media

through gentle perturbation in a water bath. The number of nodules formed on each

seedling was counted. The shoot and root system of each seedling were separated and

dried in an oven at 65°C for 48 h prior to measuring shoot dry mass (SDM) and root

dry mass (RDM).

**Statistical analysis**

All data were analyzed using SAS version 9.4 Software (SAS, Inc., Cary, NC). Differences in morphological variables among treatments were identified using PROC GLIMMIX. Type III tests of fixed effects were used to examine interactions and main effects and differences of least squares means were adjusted for multiple comparisons.

# Results

There were no statistically significant differences in initial seedling height or caliper among fertilizer treatments ($P = 0.36$ and $0.93$, respectively) or between inoculation treatments ($P = 0.43$ and $0.72$, respectively). Both weekly fertilizer rate ($P = 0.0098$) and inoculation ($P = 0.0001$) affected final seedling height. Specifically, seedlings were 28% taller when treated with the highest fertilizer rate (4 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$) compared to the lowest rate (0 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$) and 32% taller when inoculated with rhizobium compared to those uninoculated. Seedlings grown under the medium fertilizer rate (2 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$) were not significantly different than those grown under the high or low rates (Table 3.1). The main effects of fertilizer and inoculum interacted to affect relative height growth ($P = 0.02$; Table 3.1 and Figure 3.1). Seedlings displayed greater relative height growth when inoculated with rhizobium regardless of fertilizer rate and when treated with the highest fertilizer rate regardless of inoculation treatment (Figure 3.1).

Weekly fertilizer rate affected final seedling caliper ($P < 0.0001$; Table 3.1). Seedlings had greater caliper (and greater relative caliper growth) when treated with the highest fertilizer rate compared to either the low or medium rates (Table 3.1). Neither final caliper nor relative caliper growth differed significantly between inoculation treatments ($P = 0.5598$ and $0.9487$, respectively; Table 3.1).

The main effects of fertilizer and inoculum interacted to affect SDM ($P = 0.0343$; Table 3.1 and Figure 3.2). Similar to relative height growth, seedlings had a greater SDM when inoculated with rhizobium regardless of fertilizer rate and when treated with the highest fertilizer rate regardless of inoculation treatment (Figure 3.1; Figure 3.2). Seedling RDM was greater when treated with the medium or highest fertilizer rates compared to the lowest rate ($P < 0.0001$), but did not differ significantly between inoculation treatments ($P = 0.0625$; Table 3.1).

All seedlings exhibited nodulation, however the mean number of nodules was greater on seedlings treated with the medium fertilizer rate compared to the lowest rate ($P = 0.0337$) and when seeds were inoculated compared to those uninoculated ($P < 0.0001$; Table 3.1).

## Discussion

Seedlings treated with 4mg Applied Fertilizer·week$^{-1}$, and those inoculated treated 2mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$ all produced comparable height, SDM, and RDM. The similar morphology between the medium and high fertilizer rates when inoculated with rhizobium indicates that fertilizer use can be reduced while maintaining seedling size if inoculated with rhizobium. Studies on other leguminous

species have also found it possible to cultivate seedlings with a similar morphology under reduced fertilizer rates if seedlings are inoculated (Chaukiyal et al. 2013; Dumroese et al. 2009).

Notably, uninoculated seedlings still formed root nodules while growing in sterile growing media. Other studies have found nodulation to be present in the absence of rhizobium (Dumroese et al. 2009; Lesueur and Duponnois 2005), possibly due to contamination via wind or insects or while fertigating (Dumroese et al. 2009). Nonetheless, inoculated seedlings produced approximately 5 times the number of nodules compared to uninoculated seedlings. The formation of nodules in the nursery has been found to improve growth and survival once outplanted (Thrall et al. 2005). The lowest fertilizer rate likely delivered a scarcity of N to the seedling, creating a lack of nodule stimulation. Other studies have found depressed nodulation under low fertilizer regimes (Chaukiyal et al. 2013; Idieka and Odee 2005; Dumroese et al. 2009). The greatest nodule formation occurred when inoculated and provided with the medium weekly fertilizer rate. Despite inoculation, nodulation decreased under the highest weekly fertilization treatment. Higher rates of available N have been found to suppress nodulation (Saito et al. 2014; Chaukiyal et al. 2013; Goicoechea et al. 2004; Thomas et al. 2000). This suggests that 4mg fertilizer·seedling$^{-1}$·week$^{-1}$ is excessive when considering nodule formation. A decrease in nodulation can result in the forfeit of long-term benefits associated with nodulation (Thrall et al. 2005).

The optimal rate of fertilizer applied to N-fixing seedlings is dependent on the deficit between that which is fixed by rhizobium and that required by the seedling for growth (Roberts et al. 1983). Seedlings inoculated with rhizobium and under a

medium weekly fertilization rate exhibited growth similar to those receiving a high

weekly fertilizer rate while seedlings grown under 0 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$ were stunted in growth and formed less nodules regardless of inoculation.

It is possible for nurseries to fertilize black locust seedlings at a lower rate if seeds are

inoculated upon sowing while achieving the comparable nodule formation and

growth. This has important management implications: nurseries need to fertilize black

locust seedlings but can drastically reduce the amount of fertilizer used, reducing the

possibility of local surface and groundwater pollution, while achieving seedlings with

comparable morphologies if seeds are inoculated with rhizobium.

**Acknowledgment**

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# Tables

**Table 3.1.** Effects of fertilizer rate (0, 2, 4 mg Applied Fertilizer·seedling$^{-1}$·week$^{-1}$) and rhizobium inoculation on growth and development of *Robinia pseudoacacia.* Means (SE) are presented with associated Tukey-Kramer groupings within each main effect when interactions were not statistically significant.

| | | Height (cm) | Relative height growth | Caliper (mm) | Relative caliper growth | Nodules (number seedling$^{-1}$) | RDM (g) | SDM (g) |
|---|---|---|---|---|---|---|---|---|
| Fertilizer (F) | 0 mg | 6.56±0.42 B | 1.51±0.18 | 1.17±0.04 B | 0.04±0.04 B | 6.35±1.62 B | 0.04±0.01 B | 0.09±0.01 |
| | 2 mg | 7.25±0.43 AB | 1.79±0.19 | 1.24±0.04 B | 0.10±0.04 B | 12.33±1.67 A | 0.05±0.01 A | 0.12±0.01 |
| | 4 mg | 8.44±0.42 A | 2.55±0.18 | 1.44±0.04 A | 0.27±0.04 A | 10.85±1.62 AB | 0.07±0.01 A | 0.16±0.01 |
| Inoculum (I) | No | 6.38±0.35 B | 1.67±0.15 | 1.29±0.03 | 0.14±0.04 | 3.43±1.33 B | 0.05±0.01 | 0.10±0.01 |
| | Yes | 8.45±0.35 A | 2.22±0.15 | 1.27±0.03 | 0.14±0.04 | 16.26±1.35 A | 0.06±0.01 | 0.15±0.01 |
| Type III Tests of Fixed Effects | | | | | | | | |
| F | 2/53[1] | 5.06 (0.0098) | 8.82 (0.0005) | 13.81 (<0.0001) | 8.16 (0.0008) | 3.62 (0.0337) | 13.55 (<0.0001) | 9.07 (0.0004) |
| I | 1/53 | 17.70 (0.0001) | 6.88 (0.0113) | 0.34 (0.5598) | 0.00 (0.9487) | 45.89 (<0.0001) | 3.62 (0.0625) | 17.08 (0.0001) |
| F*I | 2/53 | 1.97 (0.1491) | 4.12 (0.0218) | 1.53 (0.2254) | 2.00 (0.1449) | 0.37 (0.6928) | 1.92 (0.1564) | 3.60 (0.0343) |

[1] - Degrees of Freedom

# Figures
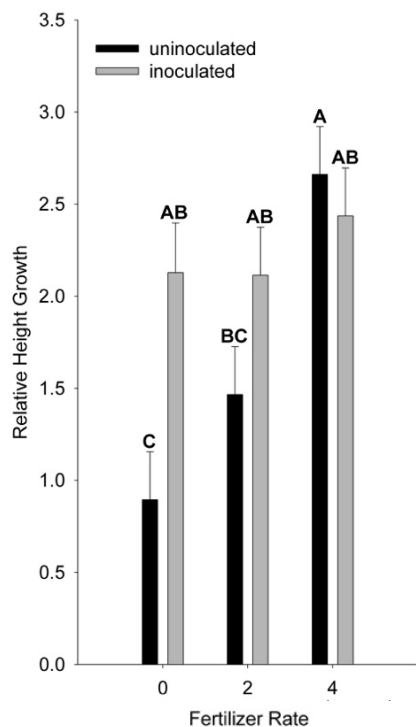


**Figure 3.1.** Mean (± SE) *Robinia pseudoacacia* seedling relative height growth across fertilizer rates (0, 2, 4 mg Applied Fertilizer•seedling$^{-1}$•week$^{-1}$) grown from uninoculated (black) and rhizobium-inoculated seed (gray bars). Different letters indicate significant differences ($\alpha = 0.05$)
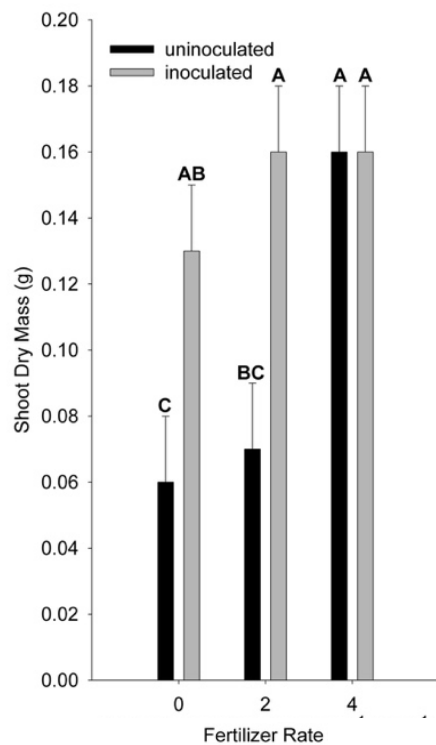
**Figure 3.2**. Mean (± SE) *Robinia pseudoacacia* seedling shoot dry mass (g) across fertilizer rates (0, 2, 4 mg Applied Fertilizer•seedling$^{-1}$•week$^{-1}$) grown from uninoculated (black) and rhizobium-inoculated seed (gray bars). Different letters indicate significant differences ($\alpha = 0.05$)

## Chapter 4: Conclusion

Artificial regeneration is a fundamental practice in renewing forests and restoring degraded ecosystems. While regeneration science has traditionally focused on restoration after timber harvest or disturbance such as fire, emphasis is now increasingly placed on restoring degraded sites to capture ecosystem services (Oliet et al. 2013; Oliet and Jacobs 2012). Moving forward, artificial regeneration using seedlings will remain an essential means to restore degraded ecosystems (Haase and Davis 2017).

Seedlings can exhibit a wide range in quality depending on seed source and nursery culture (Puértolas et al. 2012; Pinto et al. 2011), where quality is described by the seedlings ability to survive and grow once outplanted (Duryea 1984; Landis and Dumroese 2006). Cultivating seedlings is never a static process. It is always evolving to meet the demands of the "customer," which are ultimately driven by the environment. Factors including disease, mean annual rainfall and temperature, extreme weather events, current species composition, soil nutrients and quality, and browse threat are all ever-evolving phenomena we need to adapt seedling cultivation practices to.

To improve seedling quality, principals of the Target Plant Concept (TPC) should be used to guide nursery culture (Dumroese et al. 2016). This concept uses conditions of the outplanting site as a guide to create a seedling quality standard to which the nursery can adapt their practices (Haase and Davis 2017). A target seedling is cultured to achieve morphological and physiological characteristics associated with high outplanting survival and growth for a specific site (Dumroese et al. 2005).

Inherently, an important component of the TPC is the close collaboration between

nursery growers and clients who are familiar with the limitations of the planting site

(Dumroese et al. 2005). Ultimately, the characteristics of the outplanting site and the

seedling-growing regime should not be viewed individually, but instead

synergistically. Well-established and scientifically supported growing protocols

which anticipate limiting factors on the outplanting site are essential in producing

quality seedlings.

The growth form and physiology of seedlings are controlled by genetic and

environmental factors that can be manipulated in cultivation. It is typically

recommended to use a local seed source from an appropriate seed zone in restoration

(Bower et al. 2014; Ares et al 2000). Seedlings from a local seed source may exhibit

greater genetic resistance to any diseases present on the site (Wilhelmi et al. 2011)

and can be genetically predisposed to better tolerate abiotic stressors (Bingham and

Simard 2013). Phenotypic plasticity makes it possible for seedlings to employ

different compensatory mechanisms to tolerate environmental stressors. It is possible

for nurseries to prompt compensatory strategies through nursery culture (i.e. drought

hardening or nutrient loading). Decisions about the way seedlings are grown,

including seed selection, container choice, length of time grown in the nursery,

drought hardening, and fertilization regime should all be made with an understanding

of the plant's adaptive growth form to site conditions. Morphological and

physiological quality standards that are based on species-specific research under

limiting conditions can help ensure seedlings express a high fitness for purpose. This

generates a need for research to better understand how koa alters its physiological

status under low available water and P. The nursery and client should work together to establish guidelines for quality, this is commonly accomplished by setting minimum and maximum acceptable height and root-collar-diameter (Dumroese et al. 2005). Most sites in need of restoration have one or more limiting factors to seedling establishment. Identifying ecophysiological responses to stressors can be advantageous in growing seedlings able to overcome constraints to survival. Growing seedlings that are morphologically and physiologically similar to the natural adaptive phenotype should increase survival.

The goal of this thesis is to assess compensatory strategies employed by koa in conditions of low water and P, and to assess the effect of rhizobium inoculation and fertilizer interaction on black locust seedlings in a way that can reduce pollution caused by fertilization without compromising seedling morphology. The project described in chapter 2 found that though less massive, seedlings subject to reduced W or P treatments were able to employ compensatory strategies including increasing WUE or employ other physiological mechanisms to photosynthesize at a rate compared to seedlings grown with adequate available P. Understanding seedling compensatory strategies to site limiting factors has been shown to improve outplanting growth and survival. Research that continues to build on this knowledge using field trials is essential in predicting the best growing regime.

The project described in chapter 3 found that black locust seedlings were able to achieve comparable morphological characteristics under a reduced weekly fertilizer rate if seeds were inoculated with rhizobium. The simple, inexpensive, strategy of inoculating N-fixing species makes it possible to reduce fertilizer usage

and potential waste without compromising seedling development. Improving practice through reducing polluting practices makes the field more sustainable and more readily portable to developing countries where fertilizers may be more expensive and less available. Future research to continue improving black locust cultivation should focus on rhizobium inoculation and exponential fertilization. This is another strategy that reduces fertilizer waste through matching the delivered fertilizer to the demand of the seedling over the course of its life, instead of giving it equal fertilizer.

Growing seedlings for restoration should be conducted in the most sustainable way possible. Building on a strong base of knowledge gained through industrial forest regeneration practices, there are many opportunities to expand this knowledge to the specific needs and challenges found in restoration programs. Funding for ecological restoration is usually limited, addressing knowledge gaps that hinder successful seedling establishment will only increase the economic viability of these projects. As more ecological restoration projects using seedlings are successfully completed globally, the harmful effects of deforestation including the loss of biodiversity, soil erosion, loss of cultural assets, and forfeiting the economic benefits of forest products will become ameliorated. Through researching best practices, engaging in technology transfers, and generating well-trained and educated professionals, the ecological restoration industry can to capture ecosystem services and improve the economic well-being of communities in a way that transcends borders.

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