

UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ESTABLISHMENT OF A RARE RIPARIAN TREE, *ALNUS MARITIMA*

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

MASTER OF SCIENCE

By

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Norman, Oklahoma

2016

ESTABLISHMENT OF A RARE RIPARIAN TREE, *ALNUS MARITIMA*

A THESIS APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

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Acknowledgements

I would like to thank my advisor Dr. Phil Gibson for his guidance and support with research, teaching and writing. Also, thank you for allowing me to pursue my interests in conservation. A special thanks to Dr. Heather McCarthy for the insightful discussions about statistics and research, as well as her time and support that she provided. Also to Dr. Priscilla Crawford and Dr. Laura Souza for project advise.

Special thanks to Michael Kistenmacher for the encouragement to continue through the challenges of graduate school, as well as, help with data collection, problem solving and teaching me how to use R. My parents, Steve and Annette Ehardt, for emphasizing the importance of education and for support through graduate school. And to my in-laws, Hans and Monika Kistenmacher, thanks for encouragement and means to continue my education.

Jona Tucker and the Nature Conservancy for funding and allowing me to conduct research at Oka' Yanahli Blue River Preserve. Oklahoma Department of Wildlife, and Boy Scouts of America Camp Simpson for access to their property for surveys and seed collection.

Table of Contents

Acknowledgements.....	iv
List of Tables.....	vii
List of Figures.....	viii
Chapter 1: Introduction.....	1
Literature cited.....	5
Chapter 2: Population survey and species distribution models of the rare tree <i>Alnus</i> <i>maritima</i> in Oklahoma.....	7
Abstract.....	7
Introduction.....	8
Methods.....	10
Results.....	16
Discussion.....	17
Acknowledgements.....	20
Literature Cited.....	21
Tables.....	28
Figures.....	31
Chapter 3: Germination, Survival And Establishment Of A Rare Riparian Species <i>Alnus maritima</i>	41
Abstract.....	41
Introduction.....	42
Methods.....	47

Results.....	54
Discussion.....	58
Acknowledgements.....	65
Literature cited.....	65
Tables.....	76
Figures.....	77
Chapter 4: Summary.....	89
Literature Cited.....	91

List of Tables

Chapter 2

Table 1: Correlation between environmental predictor variables used in species distribution model. March Min.: average March minimum temperature, March Max.: average March maximum temperature, July Min.: average July minimum temperature, July Max...: average July maximum temperature, January Min.: average January minimum temperature, January Max.: average January maximum temperature, SWHC (25cm): average soil water holding capacity at 25 cm, TIN: time-integrated normalized difference vegetation index, SWHC (150cm): average soil water holding capacity at 150 cm.....	28
Table 2: Number of individuals counted in the area surveyed between northern and southern points for four locations: Blue River Public Fishing and Hunting Area, Pennington Creek Crossing, Pennington Creek at Tishomingo National Fish Hatchery and Boy Scout Camp Simpson.....	30

Chapter 3

Table 1: Average change \pm standard deviation in root collar diameter and height for seedlings grown in control, clipped and burned plots without (0-225 days) competitor and with (225-440) competitor.....	76
---	----

List of Figures

Chapter 2

- Figure 1: State model extent includes beige, green and grey colored counties. Local model extent includes green and grey colored counties. Inset map shows Johnston and Pontotoc counties (grey) with streams (blue lines) and *A. maritima* occurrence locations (points). Oklahoma state boundary indicated by dotted line.....31
- Figure 2: Habitat suitability values for *Alnus maritima* at the state (A) and local (B) spatial extent. Habitat suitability value is the relative probability of presence with 0.8-1 meaning a high relative probability and 0-0.2 meaning a low relative probability.....33
- Figure 3: Average receiver operator characteristic curve based on test data for the state (A) and local (B) spatial scales. Light grey line indicates mean ROC curve with standard deviation represented by dark grey shaded region. Black line represents a random prediction (i.e. AUC=0.5)35
- Figure 4: Jackknife results of environmental variable importance for the state (A) and local (B) spatial scales. Black bars represent contribution of only specified variable to the model. Light grey bar depicts model gain without specified variable. Bottom, medium grey bar is total model gain with all variables.....37
- Figure 5: Difference between Habitat suitability values for the local compared to state model for the local extent. Blue values indicate a higher suitability value

predicted by the local model compared to the state model. Red values indicate a higher suitability value predicted by the state model. Yellow values indicate similarity between the two models. Triangles represent occurrence locations.39

Chapter 3

Figure 1: (A) *Alnus maritima* regional populations with Oklahoma, Georgia, Delaware and Maryland counties that contain individuals shaded grey. Black box indicating inset map B. (B) Seed collection sites at three extant *Alnus maritima* populations (*) along the Blue River, Delaware Creek and Pennington Creek in Oklahoma. Tishomingo National Wildlife Refuge (TNWR) is the location where seeds were buried for stratification. Oka’ Yanahli Blue River Preserve, represented by triangle and circle, is the location of seedling establishment experiments. Box surrounding Oka’ Yanahli Blue River Preserve indicates inset map C. (C) Oka’ Yanahli Blue River Preserve has three islands where seedling establishment experiments were conducted. Representation of treatment plots with the northern island containing the disturbance experiment (control(A), clipped (B), and burned (C)). The two southern islands had control (R) and inoculated (I) seedlings used in the microbiome study.....77

Figure 2: Mean final number of seed germination out of 50 seeds per replicate for non-stratified (NS), 32, 64, 96 and 128 day stratified seeds under increasing temperature regime (non-shaded bars) and decreasing temperature regime (shaded bars). Error Bars represent one standard deviation. Letters above

columns distinguish significant differences ($p < 0.05$) between treatments for increasing temperature regime (non-shaded bars). No significant difference was found for decreasing temperature regime (shaded bars).79

Figure 3: Kaplan Meier Curve Analysis depicting germination proportion under increasing (A) or decreasing temperature regime (B) over time (days) with a secondary x-axis illustrating the change in temperature ($^{\circ}\text{C}$). Points represent different storage treatments (Non-stratified, 32 days, 64 days, 96 days and 128 days). Error bars represent standard error.....80

Figure 4: Hazard ratios (filled square) from Cox Proportional Hazards Model for storage treatments (32D, 64D, 96D, 128D) compared to baseline treatment (*italics*) under increasing temperature regime (A) and decreasing temperature regime (B). Bars represent 95 % confidence intervals. Dotted vertical line represents baseline hazard rate (i.e. hazard rate=1). Significance indicated by * with $p = 0.05$ (*), $p = 0.05 - 0.001$ (**) and $p < 0.001$ (***).....82

Figure 5: Kaplan-Meier Curve Analysis showing percent survival of seedlings planted in control, clipped and burned plots with time (days) since planting. Dashed line represents standard error. The vertical dotted line illustrates presence of *Strophostyles helvola* in the burn treatment.84

Figure 6: Average change in root collar diameter (A) and height (B) for control (solid line) and inoculated (dotted line) seedlings under different soil volumetric water content conditions. Shaded region illustrates the null hypothesis of equality for the nonparametric ANCOVA.85

Figure 7: The change in (A) seedling survival proportion over time (days), (B) foliar nitrogen content (%), (C) foliar N15 isotope ($\delta^{15}\text{N}$), (D) foliar carbon content (%), (E) foliar C13 isotope ($\delta^{13}\text{C}$), and (F) C: N ratio for control (open circle) and inoculated individuals (shaded triangle) before and after planting (represented by dotted horizontal line). Error bars represent standard error. Asterisk (*) represents significance ($p < 0.05$) and the cross (+) represents near significance ($p < 0.08$) 8

Chapter 1- Introduction

Species conservation is becoming increasingly important as the environmental impacts from growing human populations continue to reduce biodiversity at an alarming rate (Thomas et al 2004). To determine the best management plans for preserving biodiversity, biologists must understand the biology of rare species (Kunin and Gaston 1993). For the majority of rare plant species, the basic biological information that explains population growth and persistence is lacking (Schultz and Gerber 2002). Specifically, inadequate information about current population sizes, potential niche locations and requirements for survival at early life stages restrict management and conservation decisions and efforts. Current population sizes and the eminent threat of that population to succumb to potential extinction risks are used to determine species conservation rankings (Harmon and Braude 2010). Rare species have small populations that are susceptible to genetic drift and the loss of genetic diversity through environmental fluctuations (Thomas 1994).

The international conservation rating provided by Global Trees for *Alnus maritima* (seaside alder) is ranked as endangered because populations are genetically distinct with low genetic diversity within each population and vulnerable to impacts from human development and climate change (Shaw et al. 2014). Each population is faced with different potential threats and are given state level conservation ratings based on population size and risks that threaten the populations. The northern most population, *Alnus maritima* subsp. *maritima*, has a state conservation ranking of

critically imperiled (S1) in Maryland and imperiled status (S2) in Delaware (FWS). Current population size in Delmarva Peninsula is unknown but the population is predicted to decrease as sea levels begin to rise and urban development alters this fresh water system. *Alnus maritima* subsp. *oklahomensis* is similar to *A. maritima* subsp. *maritima*, in that the current population size is largely unknown and population threats vary from decreased water from aquifers to lack of new individuals establishing from seed, resulting in a state conservation ranking of imperiled (S2, Oklahoma Biological Survey 2014). Lastly, *Alnus maritima* subsp. *georgiensis* has a state conservation ranking of critically imperiled (S1). Having a population size of a few hundred individuals, this population has a lack of genetic diversity and exposure to runoff from neighboring agricultural lands may negatively impact current habitat (Jones and Gibson 2011, Shaw et al. 2014). Because of the large spatial separation and the genetic identity of each subspecies, Shaw et al. (2014) suggest protecting all populations because of the potential for further decrease in genetic diversity due to anthropological development and climate change. Understanding the risks and limitations of each subspecies will help conservationists determine the most efficient way to preserve this species.

The studies in the following chapters examine population size and seedling establishment for conservation of the *A. maritima* subsp. *oklahomensis* population. Because the current population size for *A. maritima* in Oklahoma is unknown, the survey in Chapter 2 provides the first estimate of populations size for populations that had been sampled for genetic analysis (Jones and Gibson 2011, 2012). Also in

this chapter an estimate of *A. maritima*'s potential niche using species distribution models was developed. Conservationists often use species distribution models to determine the potential habitat suitability, areas for surveying, diversity hotspots and potential areas for mitigation. Species distribution models are widely used to make conservation decisions (Guisan and Zimmermann 2000).

In chapter 3, the study investigates the lack of establishment of genetically new individuals at the seed and seedling life stages. Previous research found that *A. maritima* seeds have reduced total seed germination after seeds experienced -15°C and -20°C cold stratification in the laboratory (Schrader and Graves 2000a). However, these temperatures do not reflect natural temperatures, therefore, I evaluated how field stratification influenced seed germination totals and germination rate. If seeds are found to survive field stratification and germination occurs readily, then the seedling life stage potentially becomes the more important life stage hindering establishment.

Also in chapter 3, another study evaluated how altering the biotic and abiotic environment through fire and clipping influenced survival and establishment of seaside alder. I hypothesized that seedlings would have increased growth and survival in plots that experienced burning and clipped than in control plots. Because seaside alder has relatively slow shoot growth under greenhouse conditions (Schrader and Graves 2000b, Schrader et al. 2006, Gibson et al. 2008), I predicted

that fire and clipping would reduce competition above ground, resulting in increased growth and survival in burn plots compared to control plots.

Also in this chapter, this study examined the symbiotic relationship between *A. maritima* and their microbiome. For this study, individuals were inoculated with crushed nodule suspension then transplanted inoculated and reference plants into the field. I predicted that inoculated alders will have increased growth and survival compared to reference plants as seen in other greenhouse alder studies. I hypothesized the foliar nitrogen isotope signatures would be different between reference and inoculated individuals at the time of planting due to inoculated individuals having a different nitrogen source than reference plants.

These studies provide insight into the limitations of *A. maritima* establishment at the seed and seedling life stage. Further, they provide baseline population size data and potential areas for future population census studies. Through determining the factors limiting establishment and the population sizes of *A. maritima*, conservationists can make more informed management decisions which may lead to the successful preservation of this species.

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Chapter 2- Population survey and species distribution models of the rare tree *Alnus maritima* in Oklahoma

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Abstract

Climate change and increasing human impacts threaten rare, endemic plant populations through increased habitat fragmentation and loss of genetic diversity. *Alnus maritima*, the tree species with the most highly disjunct distribution in North America, is critically endangered in Oklahoma. It occurs in two Oklahoma counties, but there are no known estimates of population size or distribution within them. In this study, we provide the (i) first detailed estimates of current population size, (ii) determine areas with suitable habitat for future surveys or conservation efforts at state and local levels and (iii) identify environmental factors that determine *A. maritima*'s distribution in Oklahoma. We surveyed approximately 1.3 km² in Johnston and Pontotoc counties and counted approximately 4,842 *A. maritima* individuals. Using MaxEnt, we evaluated habitat suitability for *A. maritima* based on occurrence records and geo-referenced environmental data at the state and local

extent. The most likely suitable habitat was identified in the southern portion of Oklahoma with isolated hotspots, mostly occurring along the current known populations. March maximum temperature and landcover were the most important environmental variables for the state and local models, respectively. These results provide essential baseline population estimates and identify target areas for future conservation efforts. The methods and results from this study can be used to identify suitable habitat locations and guide conservation planning for *A. maritima* in the Delmarva Peninsula and Georgia as well.

Keywords: *Alnus maritima*, Betulaceae, distribution, MaxEnt, survey

Introduction

Species distribution and population size are two factors commonly used by biologists to determine extinction risk for a species (Rabinowitz 1981, Fiedler and Ahouse 1992). Determining these two factors can be challenging because populations can be difficult to reach and surveying large amounts of land is time consuming and costly (Fiedler and Jain 1992). As a solution to these challenges, biologist have developed Species Distribution Models (SDM) that predict distributions and highlight potential areas for occurrence, preservation, reintroduction, and surveillance (Guisan and Zimmermann 2000, Kumar and Stohlgren 2009).

Species Distribution Models associate environmental conditions with natural occurrence records, typically obtained from herbarium specimen and field surveys,

to predict probabilities of past, current, and future species distributions (Guisan and Zimmermann 2000). However, developing accurate models can be difficult because of geographical sampling bias that results from species occurrence records obtained from easily assessable locations or clustered survey locations (Ferrier et al. 2002; Engler et al. 2004). This problem is especially relevant for rare species which already have limited occurrence data. Therefore, conservation biologists have developed maximum entropy (MaxEnt) modeling methods to evaluate rare species' distributions because it is effective at generating accurate distribution predictions even in instances with few or limited distribution records (Hernandez et al. 2006; Pearson et al. 2007).

Alnus maritima (Marsh.) Muhl, ex Nutt (seaside alder, Betulaceae) is a rare, riparian tree species that is considered susceptible to extinction in the near future (Shaw et al. 2014). Despite its name, seaside alder is highly dependent on freshwater, wetland environments, characteristically growing immediately adjacent to or in muddy, waterlogged soils of rivers, streams, and swamps where it occurs. It is also highly shade intolerance (Schrader et al. 2006). *Alnus maritima* populations occur in Delaware, Maryland, Georgia, and Oklahoma, giving it the most highly disjunct distribution of any tree species in North America (Little 1975, Stibolt 1981). The regional populations are recognized as separate subspecies (Schrader and Graves 2002, 2004, Jones and Gibson 2011). *Alnus maritima* subsp. *maritima*, located in the Delmarva Peninsula of Delaware and Maryland, has a state conservation ranking of S2 indicating a risk of extinction, but remaining populations are under increasing

threat due to saltwater intrusion into their fresh water habitat (FWS, Shaw et al. 2014). *Alnus maritima* subsp. *georgiensis* grows in a lone population containing a few hundred individuals in Drummond Swamp located on private land in northwest Georgia. This subspecies has a state conservation ranking of S1 denoting it is critically imperiled, due to small size and impacts of runoff from surrounding agricultural systems (Shaw et al. 2014). The third subspecies, *Alnus maritima* subsp. *oklahomensis*, grows in south central Oklahoma. It is also has a state conservation rank of S1 and is considered critically imperiled due to decreasing quality habitat (Shaw et al. 2014). All three regional populations are experiencing declining numbers due to succession and lack of recruitment into populations from seeds (Rice and Gibson 2009, Shaw et al. 2014).

The absence of current *A. maritima* population size estimates in Oklahoma is a substantial challenge for conservation biologists trying to establish management plans for this species. To address this problem, we collected baseline data on the size of known Oklahoma populations by censusing numbers of trees on public and private lands. We combined these baseline data with geographic information obtained from herbarium specimens to build a MaxEnt distribution model to predict *A. maritima* subsp. *oklahomensis*' potential distribution in Oklahoma based on key environmental variables to guide the development of a management plans for existing populations and identify other potential locations where there may be additional, currently unknown, individuals.

Methods

Population survey. We visually surveyed known occurrence locations for seaside alder in the United States Fish and Wildlife Service Blue River Public Hunting and Fishing Area, Pennington Creek at the Tishomingo National Fish Hatchery, Pennington Creek Crossing, and the Camp Simpson Boy Scout of America property located in Johnston and Pontotoc Counties (Figure 1). Population surveys were conducted by kayak and on foot along the bank on both sides of the streams and surrounding areas in the fall of 2011. Because *Alnus maritima* grows as a multi-stemmed deciduous tree, we classified a single individual as several stems emerging from a common root clump.

MaxEnt model study area. We conducted MaxEnt distribution modeling at the state and local spatial extents to better identify ecological factors influencing seaside alder distribution and, consequently, increase overall prediction accuracy of the model (Turner 1989, Levin 1992, Wang et al. 2015). Oklahoma has three environmental gradients that are critical determinants of plant distributions (Brock et al. 1995, McPherson et al. 2007). First, there is a gradual increase in average annual precipitation from the northwest with (mean annual precipitation 50.8 cm) to the southeast with a (mean annual precipitation 127 cm). Second, average growth season length gradually decreases from southern Oklahoma (~220 days) to northern Oklahoma (~190 days). Third, there is an elevation gradient with the northwest region approximately 1,515 meters above sea level, while the southeast region is 110 meters above sea level. The statewide extent covered the majority of Oklahoma,

excluding the xeric panhandle and far western portions of the state that do not contain the wetland environments required to support *A. maritima* populations (Figure 1). The local extent analysis included two counties, Johnston and Pontotoc, that are known locations for *A. maritima* presence, and the twelve counties (McClain, Garvin, Murray, Carter, Love, Marshall, Bryan, Atoka, Coal, Hughes, Seminole and Pottawatomie) that surround them (Figure 1).

MaxEnt model occurrence data and environmental variables. Eighteen unique occurrence records for *A. maritima* were obtained from the Oklahoma Natural Heritage Inventory database and Oklahoma Vascular Plants database for analysis in the MaxEnt (version 3.3.3) distribution model (<https://www.cs.princeton.edu/~schapire/maxent/>; Phillips, et al. 2006). An additional eight occurrence records were added from population surveys.

Environmental layers were chosen based on the current knowledge of *A. maritima* biology and were assembled using ESRI ArcGIS 10.3. *Alnus maritima* is a riparian species, therefore we used a stream shapefile from Oklahoma Water Resources Board (2011) with a 320-meter buffer zone around the streams to eliminate dry areas unlikely to support alders. Average water holding capacity of soil at 25 and 150 cm were obtained from SSURGO Downloader (Soil survey staff, ArcGIS 2014). Climatic conditions which often influence plant distributions such as average maximum and minimum temperature for January, March and July, were obtained from PRISM Climate Group (PRISM Climate Group 2013). Results from prior seed

germination studies indicated March temperature was important for seed germination (see Chapter 3). January and July are the coldest and warmest months, respectively, representing the typical range of temperature for Oklahoma. Duration of greenness (length of photosynthetic activity) and time integrated normalized difference vegetation index (TIN) were obtained from USGS Remote Sensing Phenology (USGS 2015) and were used to determine differences in vegetation types. Time integrated normalized difference vegetation index is the photosynthetic activity across the entire growth season. National landcover data were acquired from the Multi-Resolution Land Classification Consortium (Homer et al. 2015) to exclude urban and xeric areas where *A. maritima* would not be found. Elevation data (30 m SRTM) were obtained from United States Geological Survey (USGS 2016) to derive slope and aspect using surface toolset in the Spatial Analyst extension in ArcGIS. All environmental variables were set to the same geographic extent and projection and resampled to 30-meter spatial resolution. Then raster files were converted to ASCII file type as specified for MaxEnt input (Phillips et al. 2006).

We tested multicollinearity between environmental variables using ENMTools (Table 1, Warren et al. 2008, 2010). If two predictor variables had a correlation of $r \geq 0.70$, one variable was removed. If multiple correlations for one variable were present, then the variable containing the largest number of correlations was retained and all correlated predictor variables were removed (Kramer-Schadt et al. 2013). The remaining variables were imported into MaxEnt.

MaxEnt species distribution model. Because there are few known *A. maritima* populations, the MaxEnt species distribution model was used to predict suitable locations for occurrence. MaxEnt determines distributions based on presence-only occurrence data and does not require confirmed absences, meaning biologist need only to provide locations where the species occurs, which is a major benefit for studying rare species located in inaccessible areas (Hernandez et al. 2006, Pearson et al. 2007). By eliminating the need for confirmed absence data, presence only models reduce additional surveying that is often strenuous and decrease the possibility of false absence data that can negatively influence the prediction of the model (Gu and Swihart 2004).

MaxEnt estimates probability of occurrence as a function of the environmental predictors for each grid cell, creating a distribution based on maximum entropy. MaxEnt is effective for determining distributions of species with a small number (i.e. 5-25) of occurrence samples (Stockwell and Peterson 2002; Elith and Leathwick 2009; Wisz et al. 2008). However, small sample sizes and the potential of sampling bias can result in geographic clustering which can influence the predictive power of the distribution model (Fourcade et al. 2014). Therefore, to test for spatial autocorrelation, we used the Average Nearest Neighbor tool in ArcGIS 10.3 to test for clustering of the 26 occurrence samples and found them to be spatially correlated ($p=0.003$, $z=-2.9897$). To reduce clustering, occurrence data were resampled with a 500 meter buffer which resulted in the removal of 5 occurrence samples due to reduce autocorrelation. Occurrence data were re-evaluated for clustering ($p=0.13$,

$z=-1.5038$). Occurrence data were then exported as .csv file and formatted according to MaxEnt requirements (Phillips et al. 2006).

Environmental data for MaxEnt were characterized as either continuous or categorical data (Phillips et al. 2006, Phillips and Dudik 2008). Because of the lack of knowledge about specific environmental thresholds that influence *A. maritima*, only linear and quadratic features were selected to reduce over-parameterization of the model (Merow et al. 2013, van Proosdij et al. 2015). A cross-validation method was used to minimize the potential effect of bias sampling from the use of herbarium occurrence data (Andersen and Beauvais 2013, Merow et al. 2013). Cross-validation is a random sub-sampling technique where 80% of the data are used to fit the model and the remaining 20% evaluates the model (Merow et al. 2013). With a 5-fold cross validation, data are split into 5 independent trials, then each trial undergoes cross validation to estimate error rate (Merow et al. 2013). Accuracy of the model to predict probability of species occurrence was determined using Area under the Curve (AUC) by the Receiver Operating Characteristic. This Receiver Operating Characteristic is the proportion of known occurrences predicted present (i.e., sensitivity) compared to proportion of known absences predicted present (1-specificity) (Peterson et al. 2008). In MaxEnt, absences are known as pseudo-absences, generated by the model and are taken from randomly chosen background pixels (Phillips et al. 2006, Phillips and Dudik 2008). Because of the use of pseudo-absences, this method is different from typical Receiver Operating Characteristic presence/absence comparisons resulting in a maximum AUC value less than one

(Wiley et al. 2003). The AUC is a value used to characterize model performance. AUC values >0.9 are deemed highly accurate, $0.7-0.9$ moderate accuracy, and <0.7 as poor accuracy (Hoffman et al. 2008). Jackknife analysis was performed to determine the importance of each environmental factor (Elith et al. 2011, Yang et al. 2013). MaxEnt model produces a habitat suitability value which is the probability of presence given that the occurrence locations have a probability of presence of 0.5 (Elith et al. 2011). In jackknife analysis, variables are run independently in the model to determine training gain and then compared to the model training gain when all variables are used to determine the contribution the variable. If the training gain is high then the variable contributed strongly to the model (Elith et al. 2011, Yang et al. 2013).

Results

Population survey. A total of 4,842 individuals were counted across the 4 survey locations (Table 2). Blue River Public Hunting and Fishing area was the largest area surveyed and had the largest population with 4,430 individuals. Along Pennington Creek, there were two areas surveyed with 203 individuals near Pennington Creek Bridge and 59 individuals at the Tishomingo National Fish Hatchery. The Camp Simpson Boy Scout Property had one small population at the southern end of Delaware Creek Reservoir that contained 146 individuals.

State MaxEnt model. Habitat suitability for *A. maritima* generally increased from north to south, with the northern portion of the state having a habitat suitability of

less than 0.2 and the southern having areas with greater than 0.6 (Figure 2A). Overall, the distribution was fragmented with three noticeable areas located in south central Oklahoma where current seaside alder populations exist and two more isolated locations in southeastern and southwestern Oklahoma where habitat may be similar. The model predicted habitat suitability for *A. maritima* with a mean AUC of 0.902 ± 0.05 (average \pm standard deviation, Figure 3A), indicating a robust, accurate model. Jackknife analysis identified March average maximum temperature and landcover as the two main factors influencing *A. maritima* distribution and provided the most information for the model with a training gain value of approximately 0.6 (Figure 4A). In contrast, factors such as aspect and soil water holding capacity at 25 cm had a training gain of zero and were not important to the model.

Local MaxEnt model. At the county level, the model identified high suitability for seaside alder in an area north of the current known distribution in Pontotoc County and to the southeast of current known distribution in Bryan and Atoka counties (Figure 2B). The mean AUC for the county level was 0.78 ± 0.096 , indicating moderate accuracy (Figure 3B). Jackknife results indicated landcover contributed the most to the model with a training gain value of 0.48 (Figure 4B). Soil particle size and soil water holding capacity at 150 cm were the second and third most important variables with approximate training gain values of 0.18 and 0.14, respectively. Aspect added zero gain to the model.

Discussion

This study provides the first estimate of the current population size for *A. maritima* in Oklahoma. Our census determined there are approximately 4,650 individuals in locations surveyed, with the vast majority growing along the Blue River (Table 2). In addition to the visual count of population size, our MaxEnt models provide the first GIS-based spatial estimates of *A. maritima* distribution in Oklahoma. *Alnus maritima* distribution is hypothesized to have been widespread across eastern North America in the past (Stibolt 1981). However, the environmental data used in the state and local models presented in this study indicate that the distribution in Oklahoma was likely more restricted. The state-level MaxEnt model indicated that the most suitable areas for seaside alder were located in southern Oklahoma, predominantly in locations surrounding the known populations in south central Oklahoma (Figure 2A). These results were supported by local-level MaxEnt model that indicated areas of high suitability were just outside of the current known *A. maritima* populations (Figure 2B). When we compared the local and state models, the predictions in habitat suitability are similar except that the local-level model shows higher habitat suitability values slightly to the north of the known populations and the state-level model has higher suitability values to the south of known populations (Figure 5). Although these models have differences in predicted habitat suitability, both models indicate a restricted range of environmental conditions where *A. maritima* could potentially grow in Oklahoma under current environmental conditions.

Climate is considered to be the most influential variable affecting plant distributions at large extents (Cao and Tang 2014). Consistent with this, our results indicate that

March maximum temperature is an important determinant of *A. maritima* distribution in Oklahoma. However, March maximum temperature was highly correlated with January minimum and maximum temperature (Table 1), which suggests all three variables may be equally important. Between January and March, *A. maritima* seeds remain ungerminated in the seed bank until temperatures reach approximately 12°C (Ehardt et al. in preparation). Therefore, these climate variables could be indicators of potential for seed and seedling survival which are the limiting phase in the seaside alder lifecycle for establishment of a new individual (Rice and Gibson 2009, Ehardt et al. in preparation).

Temperature variables were more useful in developing the state level model than for the local level model because there was very little variability in temperature. Instead, the local level model identified landcover as the most important variable in determining suitable habitat for *A. maritima*. The suitable landcover types identified by the local level model were forest and water areas, which coincide with current known seaside alder populations located along streams in riparian forested areas. This habitat is thought to be suitable because sunlight and water are sufficient to meet *A. maritima* photosynthetic demands (Schrader et al. 2006). *Alnus maritima* is a small tree with no shade tolerance. Therefore, locations with high canopy height may be unsuitable areas for growth (Schrader et al. 2006). This model could potentially be improved by including fine resolution imagery that can be used to evaluate differences in canopy height (Menges et al. 1999). Analyzing distribution at

a finer resolution can also identify other important biotic variables that could improve our understanding of the distribution of this rare species.

The greatest challenge to seaside alder conservation is locating and maintaining remaining populations. Approximately 95% of Oklahoma land is privately owned (U.S. Fish and Wildlife Service 2016), therefore our survey area is restricted and further studies should explore for existing populations on private locations. Based on our census data and distribution models, there could be an additional 1,000 individuals located on privately owned land. Our survey provides a valuable estimate of *A. maritima* population size in a protected, and minimal human influenced habitat. There are likely other *A. maritima* populations on private land that we were not able to survey. Specifically, more individuals are likely to occur along Pennington Creek, which is indicated by herbarium records, however we were unable to access some of these areas due to restrictions from landowners. Our population survey and MaxEnt models can fill in knowledge gaps about *A. maritima* through providing information about future survey locations and possible regeneration locations. The methods from this study should be conducted for Delaware, Georgia and Maryland populations to determine the current distribution of *A. maritima* and possibly to evaluate how the current human development of these locations could impact the distribution of the species.

Acknowledgements

The authors would like to thank M. Kistenmacher, P.H.C. Crawford, Oklahoma Department of Wildlife Conservation, and Boy Scouts of America Camp Simpson.

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Tables

Table 1. Correlation between environmental predictor variables used in species distribution model. March Min.: average March minimum temperature, March Max.: average March maximum temperature, July Min.: average July minimum temperature, July Max.: average July maximum temperature, January Min.: average January minimum temperature, January Max.: average January maximum temperature, SWHC (25cm): average soil water holding capacity at 25 cm, TIN: time-integrated normalized difference vegetation index, SWHC (150cm): average soil water holding capacity at 150 cm

Variable	March Min	March Max	July Min	July Max	January Min	January Max	Elevation	Duration	Aspect	Slope	SWHC (25 cm)	TIN	SWHC (150 cm)
March Min	1	0.858	0.568	0.138	0.979	0.801	-0.605	0.146	0.003	0.072	-0.085	0.081	0.024
March Max		1	0.518	0.243	0.873	0.966	-0.442	0.009	-0.005	0.009	-0.076	0.012	0.064
July Min			1	0.408	0.534	0.48	-0.232	-0.067	-0.019	-0.121	0.109	0.024	0.171
July Max				1	-0.077	0.313	0.359	-0.348	-0.025	-0.202	0.111	-0.152	0.182
January Min					1	0.85	-0.502	0.098	0.005	0.089	-0.115	0.04	0.017
January Max						1	-0.264	-0.064	-0.003	0.026	-0.109	-0.044	0.049
Elevation							1	-0.323	0.014	0.033	-0.102	-0.227	-0.123
Duration								1	-0.072	0.044	-0.133	0.746	-0.126
Aspect									1	0.05	-0.022	-0.12	-0.029
Slope										1	-0.212	-0.036	-0.214
SWHC (25 cm)											1	-0.03	0.646
TIN												1	-0.034
SWHC (150 cm)													1

Table 2. Number of individuals counted in the area surveyed between northern and southern points for four locations: Blue River Public Fishing and Hunting Area, Pennington Creek Crossing, Pennington Creek at Tishomingo National Fish Hatchery and Boy Scout Camp Simpson.

Population location	Individuals	Area Surveyed (Latitude, Longitude)		Total area surveyed (m ²)
		Northern point	Southern point	
Blue River	4,430	34.3968, -96.609414	34.31458, -96.57896	1,232,913
Pennington Creek	207	34.32318, -96.703463	34.319727, -96.70646	55,760
Tishomingo National				
Fish Hatchery	59	34.356535, -96.71001	34.35536, -96.710702	19,531
Camp Simpson	146	34.411526, -96.54498	34.410443, -96.542653	5,850

Figure 1. State model extent includes beige, green and grey colored counties. Local model extent includes green and grey colored counties. Inset map shows Johnston and Pontotoc counties (grey) with streams (blue lines) and *A. maritima* occurrence locations (points). Oklahoma state boundary indicated by dotted line.

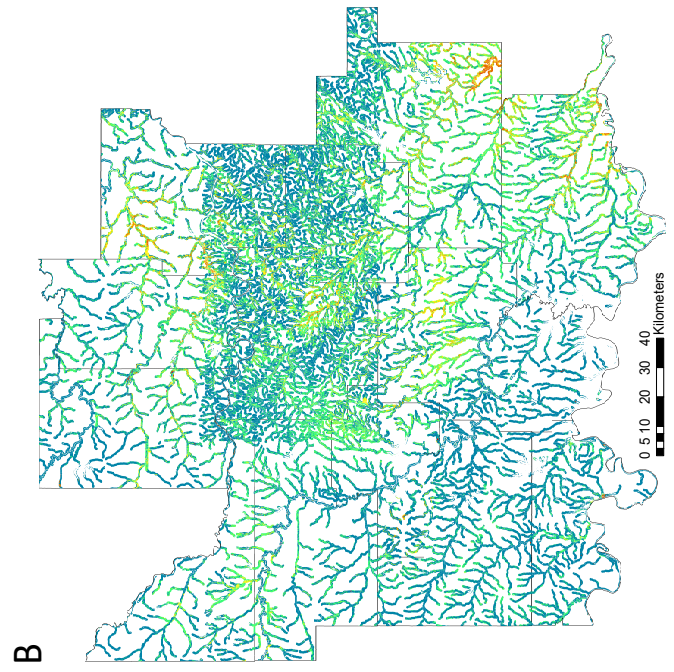
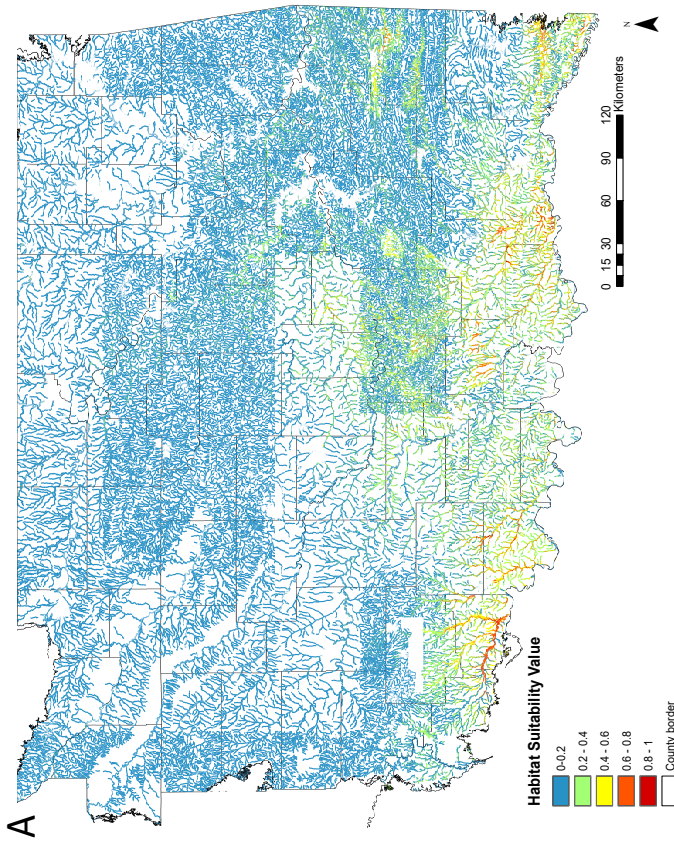


Figure 2. Habitat suitability values for *Alnus maritima* at the state (A) and local (B) spatial extent. Habitat suitability value is the relative probability of presence with 0.8-1 meaning a high relative probability and 0-0.2 meaning a low relative probability.

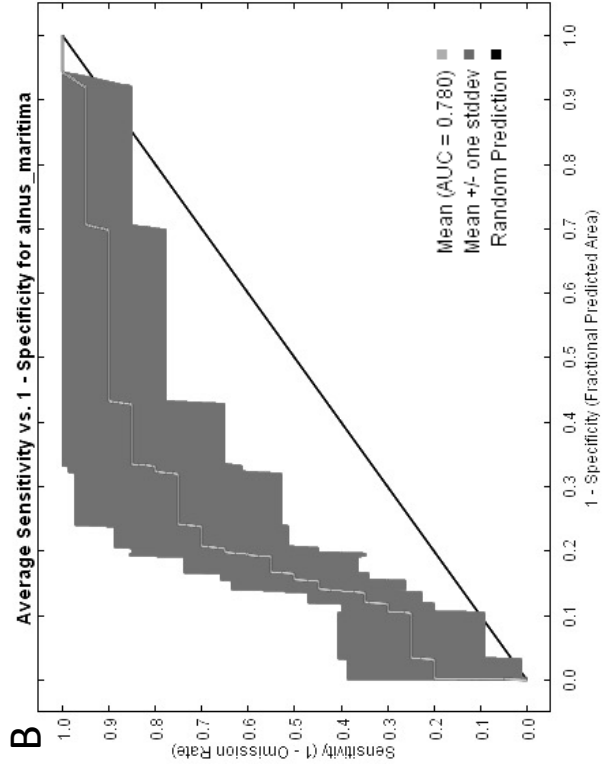
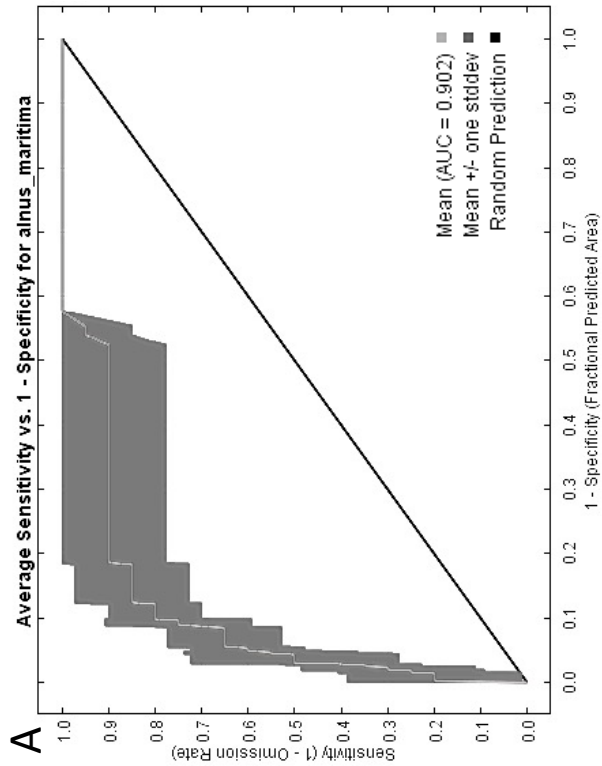


Figure 3. Average receiver operator characteristic curve based on test data for the state (A) and local (B) spatial extent. Light grey line indicates mean ROC curve with standard deviation represented by dark grey shaded region. Black line represents a random prediction (i.e. AUC=0.5).

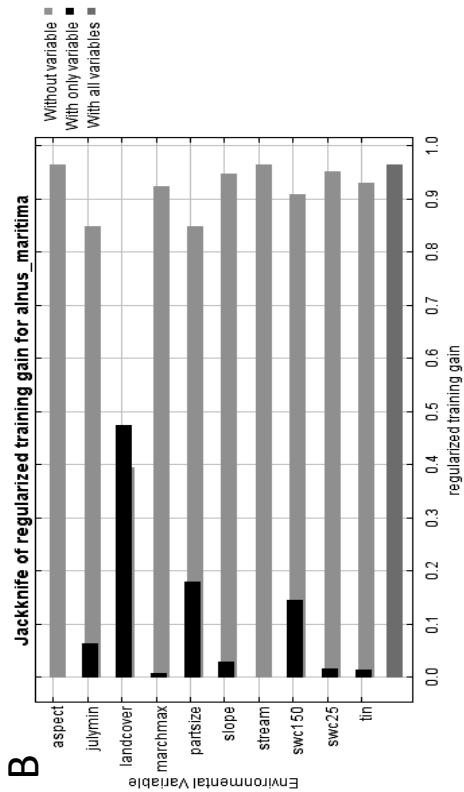
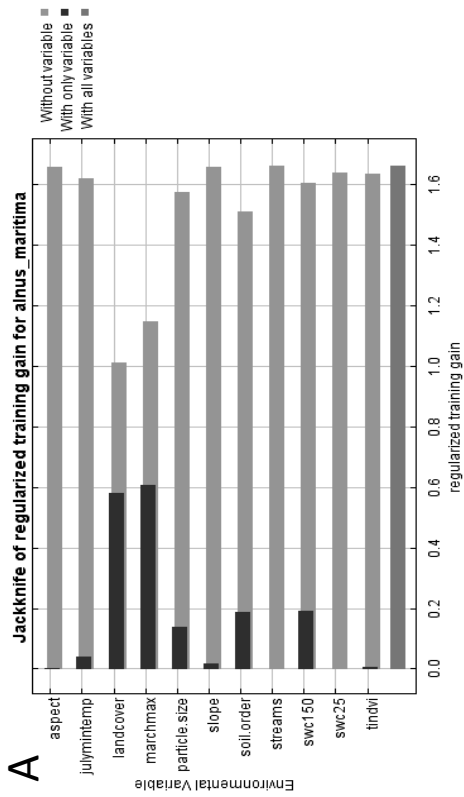


Figure 4. Jackknife results of environmental variable importance for the state (A) and local (B) spatial extent. Black bars represent contribution of only specified variable to the model. Light grey bar depicts model gain without specified variable. Bottom, medium grey bar is total model gain with all variables.

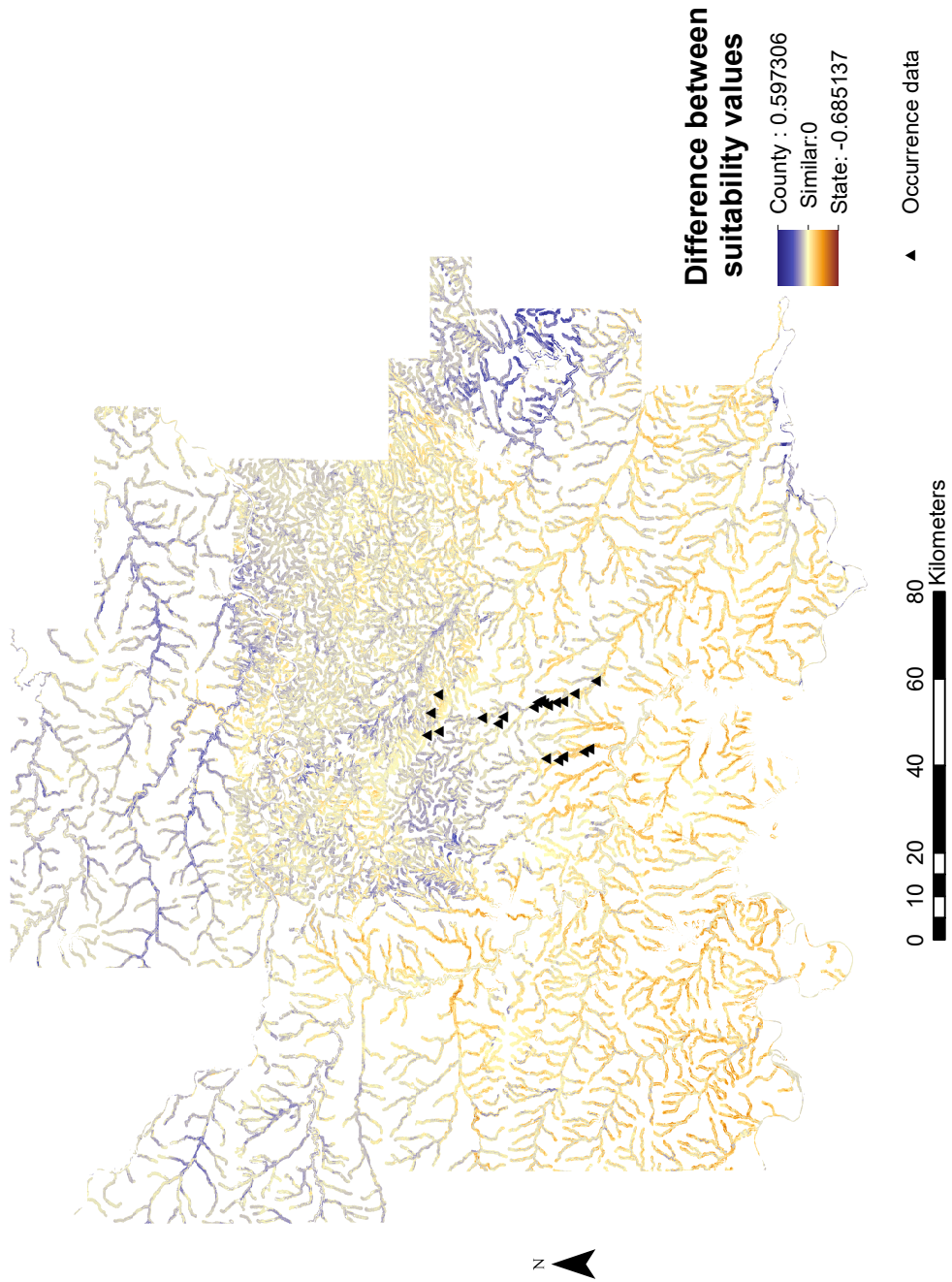


Figure 5. Difference between habitat suitability values for the local compared to state model for the local extent. Blue values indicate a higher suitability value predicted by the local model compared to the state model. Red values indicate a higher suitability value predicted by the state model. Yellow values indicate similarity between the two models. Triangles represent occurrence locations.

Chapter 3- Germination, Survival And Establishment Of A Rare Riparian Species *Alnus maritima*

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To be submitted to *Biodiversity and Conservation*

Abstract

Alnus maritima is a rare, riparian tree species showing no recruitment from seeds. We conducted three studies to identify factors potentially limiting establishment during seed and seedling stages. First, we studied the effect of field stratification to determine if low winter temperatures decrease seed viability or otherwise alter seed dormancy. Second, because *A. maritima* is an early successional species that requires high light environments, we investigated seedling establishment after burning or clipping vegetation at study sites. Third, we compared how presence or absence of the native root microbiome associated with *A. maritima* nodules influenced survival, growth, and establishment of seedlings. There were no differences in mean total seed germination between stratified seeds and non-stratified seeds, but longer periods of

stratification promoted earlier seed germination, indicating a non-deep physiological dormancy. Neither burning nor clipping influenced seedling growth or survival initially. However, seedling survival decreased in burned plots due to the release of an aggressively growing competitor. Surprisingly, seedlings inoculated with the native microbiome had reduced survival compared to uninoculated individuals, and uninoculated individuals showed a decline in survivorship after natural inoculation in the field, suggesting that the microbiological “mutualists” may be a physiological stress on seedlings during establishment. These results indicate that seedling survival and not seed viability is limiting establishment of this species.

Keywords: Betulaceae, carbon stress, conditional dormancy, disjunct distribution, nodule, stratification

Introduction

Seaside alder (*Alnus maritima*, Betulaceae) is an extremely rare, riparian species with a G3 global conservation status, indicating it is threatened and faces a risk of extinction throughout its range (Shaw et al. 2014). It has the most highly disjunct distribution of any tree species in North America (Little 1971). There are three regional populations in Maryland, Georgia, and Oklahoma (Figure 1A) that are remnants of a once larger distribution that retracted following the Pleistocene glacial period (Furlow 1979; Stibolt 1981, Schrader and Graves 2000a, 2002, 2004; Gibson et al. 2008; Jones and Gibson 2011). *Alnus maritima* subsp. *maritima* is scattered in small clusters along the Nanticoke River and Marshyhope Creek in the Delmarva

Peninsula and has a state conservation ranking of S3, indicating a moderate extinction risk (Maryland Department of Natural Resources 2016). The Georgia subspecies, *A. maritima* subsp. *georgiensis*, is a single population in Drummond Swamp located in Bartow County in northwest Georgia. This population is critically imperiled and has an S1-ranking indicating it faces a very high risk of extinction (World Conservation Monitoring Centre 1998; Chafin 2007). In Oklahoma, *A. maritima* subsp. *oklahomensis* grows in several small populations located along spring fed tributaries of the Blue River and Pennington Creek in Johnston and Pontotoc Counties. The Oklahoma subspecies is critically endangered with a high risk of extinction (state conservation status S1, Shaw et al. 2014).

Despite production of numerous viable seeds, there is no establishment of new individuals from seed in any seaside alder population (Rice and Gibson 2009; J.P. Gibson and S.A. Rice personal observations). Consequently, vegetative root sprouting from established plants is the only means of adding “new” individuals to populations. This places the species at increased risk to not only lose unique, regional, genetic variation, but also extinction as adults die in the remaining populations. Successful conservation and management of rare plant species requires basic knowledge about seed dormancy and germination and seedling establishment ecology (Schemske et al. 1994; Primack and Drayton 1997; Kolb and Barsch 2010; Godefroid et al. 2011; Copete et al. 2015). Because the seed and seedling stages are common bottlenecks in the establishment of temperate, woody species in general, and are undoubtedly an issue for seaside alder in particular, we conducted a detailed

study of seed and seedling ecology to identify potential reasons for the absence of new individuals establishing from seed in this unique, riparian tree.

Decreased seed viability and germination have been proposed as explanations for low seedling recruitment in seaside alder. Schrader and Graves (2000b), suggested that low winter temperatures kill seeds, thereby reducing seedling establishment. However, *A. maritima* may be similar to other alders and have either non-dormant or conditionally dormant seeds that would benefit from exposure to cold temperatures (Baskin and Baskin 2014). Conditional dormancy is a form of physiological dormancy in which seeds are dormant or have a narrow range of conditions within which germination can occur immediately after they mature. Exposure to specific environmental stimuli in the soil, such as moisture and temperatures between 0 and 10⁰C, cold stratify seeds and alter their physiological features causing either a shift from dormant to non-dormant or broaden the range of threshold conditions to initiate germination (Baskin and Baskin 1998, Allen et al. 2007, Baskin and Baskin 2014). Conditional dormancy has been identified in many weedy, and agricultural species (Bouwmeester and Karssen 1992; Baskin and Baskin 1998, 2000, 2014) but has not been thoroughly investigated for woody perennial species particularly in a conservation context (Baskin and Baskin 2014; Cao et al. 2014; Copete et al. 2015).

Following germination, seedling establishment is shaped by the abiotic environment and interactions, particularly competition, with other species in the community (Helenuum 1998; Kolar and Lodge 2001). Like other alders, *A. maritima* has traits

characteristic of early successional, riparian species (Dellasala et al. 2004). Because human activities have altered natural disturbance regimes in riparian environments, there is decreased occurrence of open habitats with sufficiently high water and light necessary for growth of adult seaside alders and increased competition with later successional species (Folk et al. 2004; Schrader et al. 2006; Rice and Gibson 2009). Management strategies that simulate natural disturbances and reduce competition with later successional species have increased seedling survival of rare species in riparian systems and may be necessary for seaside alder seedlings (Whisenant 1999; Dwire and Kaufman 2003; Richardson et al. 2007; Arkle and Pilloid 2009; Godefroid et al. 2011).

Competitive ability and establishment in nutrient-poor, riparian environments can be increased through mutualisms that form between alder roots and the soil microbiome (So et al. 2011; Kennedy et al. 2015). *Alnus* species frequently form symbiotic relationships with arbuscular ectomycorrhizae (Molina et al. 1994; Kennedy and Hill 2010; Kennedy et al. 2015) and nitrogen fixing bacteria in the genus *Frankia* (Tjepkema et al. 1986; Baker and Schwintzer 1990; Huss- Danell 1997; Dawson 2008). Because alders often grow in low nutrient environments, associations with the root microbiome provide plants with essential limited nutrients and increase plant growth compared to individuals without the root symbionts (Bissonnette et al. 2014). For example, symbiotic *Frankia* that form nodules on *Alnus* roots can supply 70-100% of the plant's nitrogen (Hurd et al. 2001), and arbuscular ectomycorrhiza

provide phosphorus to plants in exchange for carbon and nitrogen (Arnebrant et al. 1993; Huggins et al. 2014; Yamanaka et al. 2003).

We conducted the study described here to identify factors inhibiting establishment of seaside alder individuals from seed on Oklahoma populations. We conducted a series of field-based experiments to specifically investigate *i)* the influence of the seed bank environment on seed dormancy and germination, *ii)* the impact of disturbance on seedling establishment, and *iii)* the effect of mutualistic relationships that develop between seaside alder roots and the soil microbiome. The germination study will clarify how cold stratification affects seed mortality, dormancy, and germination in seaside alder under *in situ* seedbank conditions. If Schrader and Graves' (2000b) hypothesis is correct that exposure to low temperatures is causing high seed mortality and preventing establishment, then seeds overwintering under natural soil conditions will have lower germination than newly ripened seeds. However, if seeds are conditionally dormant, stratification in the soil seed bank should promote higher germination over a broader range of conditions than fresh seed (Baskin and Baskin 1998; Benech-Arnold et al. 2000; Allen et al. 2007; Baskin and Baskin 2014). To investigate the role of disturbance on seedling establishment, we followed growth and survival of transplanted seedlings in undisturbed, burned, and clipped plots. Given the weak competitive ability and extreme shade intolerance of seaside alder (Schrader et al. 2006), reduction in competitors through clipping or fire should result in greater growth and higher survival of plants than in undisturbed conditions. And lastly, we studied the role of associations between *A. maritima* and its root

microbiome by comparing how seedling survival, growth and leaf chemistry were affected by the presence or absence of the native, root microflora. Because alders commonly form nodules and mycorrhizal associations, we predicted that seedlings inoculated with a nodule suspension prior to planting will have increased growth and survival compared to uninoculated individuals. The results from these studies will not only provide insights on the reproductive ecology of seaside alder, but also identify factors limiting establishment of new trees that will assist in developing conservation and management strategies for this species.

METHODS

Seed Dormancy and Germination. Similar to other alders, *A. maritima* is monoecious and produces unisexual catkins. However, unlike all other North American species, which are in the subgenus *Alnus*, seaside alder is the lone member of the subgenus *Clethropsis* to grow outside of Asia (Chen and Li 2004). Members of *Clethropsis* have a distinct reproductive phenology in which they flower in the fall while leaves are still present, mature embryos over winter, and retain seeds in catkins until they are released the following fall. Alders in subgenus *Alnus* flower before leaf flush in the spring and disperse seeds in the fall of the same year (Chen and Li 2004; Schrader and Graves 2002). We collected seeds for germination studies in October 2012 at the Pennington Creek Crossing (34.322279, -96.705952), the Boy Scouts of America Camp Simpson on Delaware Creek (34.410707, -96.543099), and the United States Fish and Wildlife Service Blue River Public Fishing and Hunting Area

(34.323732, -96.593513) in Johnston County, OK (Figure 1B). Mature but unopened catkins were collected from twenty individuals in each population.

Catkins were stored in envelopes and transported to the lab to manually extract seeds. Seeds were pooled and sorted into 7.6 cm by 7.6 cm bridal veil bags with approximately 350 seeds per bag. Six bags were assigned to each treatment: non-stratified (designated NS), 32-day stored seeds (designated 32D), 64-day stored seeds (designated 64D), 96-day stored seeds (designated 96D), 128-stored seeds (designated 128D), and 160-stored seeds (designated 160D). Seeds were buried at a depth of 20 cm at Tishomingo National Wildlife (TNWR), 15 miles south of the known *A. maritima* locations (Figure 1B). TNWR has a mean annual precipitation of 98.7 cm and average 30 cm soil temperatures during winter and early spring is 14.1°C for November, 8.9°C for December, 7.12°C for January, 8.0°C for February, 11.8°C for March and 15.7°C for April (McPherson et al. 2007; Brock et al. 1995).

Germination trials were conducted under temperature regimes mimicking increasing spring temperatures and decreasing fall temperatures to evaluate conditional dormancy states (Baskin and Baskin 2014). Germination under an increasing temperature regime will identify the low temperature threshold for germination and the decreasing temperature regime will indicate the high temperature threshold. Differences in germination thresholds between non-stratified and stratified seeds, therefore, indicate a change in conditional dormancy status of the seeds (Washitani 1987; Batla and Benich Arnold 2003; Baskin and Baskin 2014). The increasing

temperature regime started at 4°C and was increased by 4°C every 4 days until reaching the maximum temperature of 32°C. Conversely, the decreasing temperature regime started at 32°C and decreased 4°C every 4 days until reaching 4°C (Batla and Benich Arnold 2003; Washitani 1987). Germination trials were conducted in Precision Model 818 Low Temperature Illuminated Incubators (Thermo Electron Corporation, Marietta, OH) on a 12 hr. light/dark cycle.

For the germination trials, six Petri dishes containing two pieces of Whatman® #1 9 cm filter paper were prepared to test each stratification treatment. Fifty seeds were placed in each Petri dish and moistened with 4 mL of deionized, distilled water. Three replicates were placed in the incubators under increasing temperature regime and three were placed in incubators under a decreasing temperature regime. During germination trials, seeds were monitored every 2 days for germination, indicated by the radicle protruding through the seed coat. At the end of each temperature regime, seeds were dissected and visually checked for white solid embryos, an indication of viability based off a previous tetrazolium test that revealed brown solid or white nonsolid embryos were not viable (Baskin and Baskin 1998; Schrader and Graves 2000a, 2000b; P.J. Gibson and C. Ehardt-Kistenmacher unpublished). Germination trials using non-stratified seeds began on November 1, 2012. Seeds for stratification treatments were buried November 2, 2012, and, a bag was retrieved from the storage location every 32 days to begin the next a germination trial.

We compared germination between stratified and non-stratified seeds using ANOVA with Tukey's HSD post hoc test in R (R Core Team 2014). Kaplan-Meier curves were used to graphically display differences in the proportion of seeds germinated between treatments. To compare germination rates, we used an Extended Cox Proportional Hazards Model with time independent heaviside functions in R (R Core Team 2014) to calculate Hazard Ratios (HR, Kleinbaum and Klein 2012; Kistenmacher and Gibson 2016). The HR is the ratio between the baseline treatment germination rate compared to the experimental treatment germination rate. The ratio of rates gives the conditional probability of an event occurring during a given time interval due to a treatment. For example, if stratified and non-stratified seeds both have one seed germinate over a given time interval, then $HR = 1$ indicating no difference in the likelihood of seed germination between the two groups. However, if 20 seeds/time interval germinate from the stratified seeds and only one seed germinates from non-stratified seeds, then $HR = 20$ indicating the stratified seeds are 20 times more likely to germinate than non-stratified seeds. The Cox Proportional Hazard Model assumption of parallelism was checked for crossing of the germination curves using a Kaplan-Meier curve analysis function *Survfit* in the R package "Survival". If the model assumption for parallelism fails, then heaviside functions (Hv) were used to split data and compare between groups before and after the curves intersect. The output after Hv contains a before and after HR (Kleinbaum and Klein 2012).

Seedling establishment. For both seedling establishment studies, ten catkins were collected from 25 individuals at the Blue River population, planted in flats containing Metro Mix potting soil, and grown under greenhouse conditions. Plants were watered daily and fertilized biweekly with Jack's Professional® (20-20-20) Balanced Water Soluble Fertilizer until plants were used in disturbance or microbiome experiments.

We conducted disturbance experiments on a 14 m X 28 m island in the Blue River at the Nature Conservancy's Oka' Yanahli Blue River Preserve (34.43852, -96.62781, Figure 1B & 1C). The island was divided into control, clipped and burned treatment zones with six 4m X 4m plots in each zone (Figure 1C). In August 2013, vegetation in both the clipped and burned plots was clipped but not removed. Fuel load and fire intensity was increased in burned plots by scattering a hay bale over plots. On September 6th 2013, the Nature Conservancy burned the southern part of the island. A barrier fence was installed to prevent large herbivores from damaging the transplants. Ten-month-old seedlings were planted on September 15th 2013. They had an average height of 49.23 ± 17.85 cm (average \pm standard deviation) and average stem diameter of 9.04 ± 5.12 mm. We planted 8 seedlings in each plot yielding 48 seedlings per treatment and a total of 144 seedlings overall planted on the island. Seedlings were monitored for survival, root collar diameter, and height using digital calipers (Pittsburgh ®). At planting, the soil volumetric water content (SVWC) was measured at 20 cm deep was measured using a HydroSense II®

(CS658 rods) from the east and west ends of each plot to account for differences in growth attributed to water availability.

To evaluate how the microbiota associated with seaside alder roots influences establishment, 144 seedlings were raised for nine months under greenhouse conditions. After 7.5 months, seedlings were randomly assigned to uninoculated reference (i.e., control) and inoculation treatment groups. To prepare the inoculation suspension, nodules were collected from adult trees in the Blue River population and returned to the laboratory. Within 24 hours after collection, nodules were washed with distilled, deionized water, and then 3-5 nodules with associated root tissue were macerated in 15 mL distilled deionized water using a mortar and pestle (Rosbrook 1990; Hurd et al. 2001). This process was repeated until 750 mL of nodule suspension was produced. In June 2013, seedlings were transplanted from flats into 15.24 cm pots. During transplanting, plants in the inoculation treatment group received ten milliliters of crushed nodule suspension around the base of the plant and plants in the control group received 10 mL deionized water.

In August 2013, the nine-month-old control and inoculated seedlings were planted on two adjacent islands in the Blue River at the Nature Conservancy Oka' Yanahli Blue River Preserve (Figure 1C). These islands were selected because they contained no established tree vegetation and were of similar size (approximately 14m x 14m). Islands were cleared of herbaceous vegetation and seedlings were planted in late August 2013. Each island consisted of four 1.5 m by 3 m plots per treatment (Figure

1C). Nine seedlings were planted within each plot for a total of 36 seedlings per treatment on each island. As in the disturbance experiment, fencing was installed to exclude large herbivores. Height and root collar diameter measurements were conducted at planting and the following spring using digital calipers (Pittsburgh ®), May 2014. Seedlings were monitored for mortality events at 7, 14, 21, 30, 40, 270 days after planting.

To further evaluate the effect of the root microbiome on seedling growth and establishment, we analyzed leaf chemistry (%C, %N, C/N) and natural abundance of foliar carbon ($\delta^{13}\text{C}$) and nitrogen isotopes ($\delta^{15}\text{N}$). At each collection date, a lower, middle and upper canopy leaf was collected from 24 seedlings per treatment. Leaves for isotope analysis were collected before inoculation, a day before planting in the field, and at one week, four weeks and eight months after field planting. To keep carbon and nitrogen values stable, leaf samples were stored on ice during transport from the field site to the lab. In the lab, leaves were dried at 60°C for 3 days in a forced air oven (Shel Lab®), then ground to a fine powder using a Retsch MM200® grinder. For each sample, approximately 2mg of ground leaf material was weighed out using a Sartorius Microbalance (CPA2P®), and packaged in a 4 X 6 mm tin capsule. Samples were analyzed at the Purdue Stable Isotope Facility using a continuous flow EA-IRMS (Sercon 20-20, SerCon Ltd, Crewe, UK) with measurement precision of 0.2‰ for $\delta^{13}\text{C}$ and 0.3‰ for $\delta^{15}\text{N}$. Isotope ratios are expressed in conventional δ notation and referenced to the PDB standard for $\delta^{13}\text{C}$ and the atmospheric standard for $\delta^{15}\text{N}$.

A Cox Proportional Hazards Model was used to determine if there was a difference in seedling survival among disturbance treatments and between inoculated and uninoculated plants. A native vine species, *Strophostyles helvola*, spread rapidly and dominated plots approximately 225 days after planting. Because of this aggressive competitor, data for the disturbance study were divided into two time periods, prior (0-225 days) and post (225-440 days) *S. helvola* competition. Heaviside functions were then used to calculate hazard ratios prior to and after 225 days. To quantify seedling growth in both the disturbance and microbiome experiments, correlation analysis was used to determine the relationship between root collar diameter and height. A nonparametric ANCOVA was also conducted using *sm.ancova* function in the R package “sm” to evaluate the differences in growth (root collar diameter, height) with initial average volumetric water content per plot as a covariate for the disturbance and microbiome experiments.

A nonlinear mixed-effects model was used in the microbiome study to compare the differences in foliar total nitrogen (% N), foliar total carbon (%C), $\delta^{15}\text{N}$ values, $\delta^{13}\text{C}$ values, and C/N ratio between inoculated and control plants. Leaf chemistry sample data were analyzed using the “nlme” package with multiple comparisons (“multcomp” package) with a Bonferroni correction in R.

Results

Seed germination. The highest mean final number of seeds that germinated under increasing temperatures occurred in 32D stratified seeds followed by the 64D seeds (Figure 2). Mean final number of seed germination declined as storage time increased resulting in the lowest mean final germination occurring in 128D seeds. Germination analysis could not be conducted on the 160D treatment because the majority of viable seeds germinated during storage, leaving insufficient seeds for germination trials. There was an overall significant difference in final mean germination among treatments (ANOVA, $df=4$, $F=5.437$, $p=0.0137$, Figure 2), but the only significant differences detected in post hoc pairwise analyses via Tukey's HSD were between 32D seeds and seed in the 96D ($p= 0.048$) and 128D ($p=0.01$) treatments.

Under the decreasing temperature regime, germination likewise declined with longer storage times (Figure 2). There was no difference in mean final number of seed germination between treatments in the decreasing temperatures (ANOVA, $df=4$, $F=2.201$, $p=0.142$).

Under increasing temperatures, germination occurred between 8°C to 24°C (Figure 3A). Seeds stratified for longer periods of time began germinating at lower temperatures. The 96D seeds had significantly higher HR than all other treatments, ranging from 2.4- 17 times more likely to germinate (Figure 4A). The 128D seeds had higher HR than the 64D, 32D and NS seeds. The 64D and 32D seeds had similar HR and both had higher HR than the NS seeds.

In the decreasing temperature regime, all germination occurred between 32°C and 28°C (Figure 3B). Field stratified seeds were more likely to initiate germination at 32°C compared to non-stratified seeds. The NS seeds had a significantly lower HR than all other treatments (Figure 4B). The 32D seeds had a lower HR than the 96D and 128D seeds but a similar HR to the 64D seeds. The 64D, 96D and 128D had no difference in HR.

Seedling establishment and disturbance. Seedlings from control, clipped and burned plots showed similar survival during first the 225 days, with only one individual dying in the burn plot (Figure 5). At the final measurement (440 days), seedling survival in burn plots decreased to 80% in comparison to control and clipped plots where all seedlings survived. Burn plot seedlings had a significantly lower likelihood of survival compared to control and clipped plot seedlings (HR=1.82e⁺⁹, CI= 6.870e⁺⁸ - 4.849e⁺⁹, $p < 0.001$).

Within the first 225 days (without competitor), there were no growth differences in seedling root collar diameter or height between the burn, clipped or control plots (non-parametric ANCOVA, $p = 0.4242$, $p = 0.9423$) when analyzed with SVWC. The average increase in growth of root collar diameter for seedlings in the burned plots and clipped plots was similar to the control plots (Table 1). Average change in height for seedlings from burn plots and clipped plots was comparable to the control seedlings (Table 1). From 225-440 days (with competitor), there was no difference in

average height ($p= 0.7473$) and root collar diameter ($p= 0.247$) between control, clip and burn treatments in surviving seedlings. Burned plot seedlings had the smallest average increase in root collar diameter, followed by the clipped plot seedlings, while the control seedlings had the largest average increase in root collar diameter (Table 1). However, seedlings in burned plots had the largest increase in average height, in comparison to seedlings from clipped plots and control plots (Table 1).

Seedling establishment and root microbiome. At the end of the experiment, the control plants had greater growth in root collar diameter than inoculated plants with SVWC included as a covariate (non-parametric ANCOVA, $p= 0.0004$, Figure 6A). Average height was not different between inoculated and control plants when analyzed with SVWC as a covariate ($p = 0.3802$, Figure 6B). Inoculated seedlings had significantly lower survival within the first 30 days after planting compared to control plants (HR=9.571, $p=0.028$, Figure 7A). However, 270 days after planting, there was no difference in survival between control and inoculated individuals (HR= 1.403, $p=0.403$).

Total foliar nitrogen (%N) was not significantly different between treatments. ($F=0.864$, $p=0.49$, Figure 7B), however, there was a significant difference in foliar $\delta^{15}\text{N}$ ($F=3.0265$, $p=0.025$, Figure 7C). Foliar $\delta^{15}\text{N}$ was similar prior to inoculation ($z=0.03$, $p=1$), but two months after inoculation, $\delta^{15}\text{N}$ content of control plants was significantly higher than in inoculated plants ($z=3.481$, $p=0.023$). After planting in

the field, foliar $\delta^{15}\text{N}$ of control and inoculated plants converged and were not different for the remainder of the experiment.

Total foliar carbon was significantly different between treatments ($F=15.47$, $p=0.001$, Figure 7D). Prior to inoculation, control plants had higher total carbon compared to the treatment plants ($z=4.051$, $p=0.002$). Prior to planting and seven days after planting, inoculated plants had higher carbon totals, however these were not significantly different ($z= -3.095$, $p=0.088$ and $z= -3.118$, $p= 0.0819$ respectively). From 7 days to 32 days, control plants increased in total carbon from ~44% to ~46% however inoculated plants stayed similar to the previous %C sample (~45%). Thirty-two days after planting and the following spring there were no differences in %C ($z=2.108$, $p= 1$ and $z=2.33$, $p=0.88$). Foliar $\delta^{13}\text{C}$ did not differ between treatments over the sampling periods ($F= 1.7$, $p=0.159$, Figure 7E). There was no significant difference in C: N ratios between treatments over sampling time for the ($F=108.3$, $p=<0.001$, Figure 7F).

Discussion

The absence of seedling establishment in *Alnus maritima* is not due to high seed mortality or inhibited germination, but rather is likely due to poor competitive ability and, surprisingly, negative impacts of the root microbiome typically associated with alders. In contrast to results from Schrader and Graves (2000b), our germination studies showed no negative impacts of seeds experiencing low temperatures. This is not surprising given that their laboratory cold stratification experiments exposed

seeds to extreme cold temperatures not experienced in Oklahoma. Our *in situ* seed bank studies demonstrated that *Alnus maritima* seeds are capable of surviving soil temperatures experienced in the field. Furthermore, field stratification broadened the low and high temperature thresholds for germination indicating that that seeds possess non-deep conditional physiological dormancy that would promote spring germination (Baskin and Baskin 2014). Therefore, seed mortality in the seed bank and inhibited germination are not likely the factors limiting *A. maritima* establishment from seed in the field.

Ecologically, seeds are viable after winter soil storage and will germinate in spring, similar to other fall seed dispersing temperate species. However, the early seedling life stage may be limiting the establishment of new individuals due to the inability of seedlings to deal with stress from above and below ground competition. Our field studies indicate that low seedling and sapling survival are likely the factors inhibiting establishment of new seaside alder individuals. Older seedling survival did not benefit from alteration of the above ground community via clipping or burning. However, survival was negatively influenced by a fast growing annual vine species, *Strophostyles helvola*, indicating seaside alder's inability to compete with this species. Our study also found that seedlings without the microbial community had similar foliar nitrogen, increased total foliar carbon, increased root collar diameter and a greater chance of survival within the first 30 days after transplantation than plants with the microbiome, suggesting that seaside alder seedlings may be incapable

of continuing necessary growth and maintenance for survival while providing adequate carbon to root symbionts such as *Frankia* and mycorrhizae.

Seed persistence and germination. Field stratified seeds have similar germination totals as fresh seeds, which opposes previous suggestions that winter air temperatures reduce total germination percentages through seed death. Schrader and Graves (2000b) implemented cold stratification conditions consisting of a three day -15 °C freeze, which is approximately -10 °C colder than Oklahoma's average minimum air temperature (Brock et al. 1995; McPherson et al. 2007). In addition, seeds are potentially buffered from extreme air temperatures through dispersal into moist soil, which is often 1-3 °C warmer (Brock et al. 1995; McPherson et al. 2007; Fernández-Pascual et al. 2015). Through burying seeds, our study more accurately represents the typical seed bank conditions and how the natural soil temperature and soil moisture conditions influence seed survival and germination. Our germination curves show that *Alnus maritima* seeds display a conditional dormancy (non deep physiological), where cold stratification results in a broadening of temperature thresholds, indicated by the lower temperature needed for germination in the seeds stratified for 96 and 128 days in the increasing temperature regime and the germination events at higher temperatures in the decreasing temperature regime (Baskin and Baskin 2004, 2014).

Seedling establishment after disturbance. After fire, alders are often among the first colonizers, because fire provides exposed substrates and high light environments for

alder establishment (Haeussler et al. 1990; Stickney 1990; Matthews 1992; Miller 2000; Lantz et al. 2010). However, in the present study, there was no evidence for enhanced growth in height and root collar diameter in surviving individuals in burned or clipped plots. Additionally, seedlings in burned plots had reduced survival due to competition with *Strophostyles helvola*. Burning initially altered the above ground community through reducing biomass of competitors, such as *Equisetum*. However, in late spring, seeds in the seed bank germinated, releasing the fast growing native herbaceous vine species, *S. helvola*, which grew on and over the high light requiring seedlings, eventually leading to mortality through shading out. Therefore, alteration of the above ground community through fire was not beneficial for establishment of *A. maritima* transplants.

Seedling establishment with root symbionts. Many plant species form symbiotic relationships with bacteria and may require pre-planting inoculation for successful establishment (So et al. 2011). Our study found no benefit from pre-inoculation with a nodule suspension. Total foliar N values were not different between inoculated and control seedlings throughout the experiment. There was a difference in isotope signatures of $\delta^{15}\text{N}$ in inoculated plants compared with control plants at planting, suggesting that nitrogen was acquired from differing sources (Shearer et al. 1978; Domenach et al. 1989; Kurdali et al. 1990; Boddey et al. 2000). However, further investigation is required to determine the amount of nitrogen derived from the soil versus a nitrogen fixating symbiont (Boddey et al. 2000). Therefore, we conclude that nitrogen demand in young seedlings may be small and there is no indication of

benefits from symbiotic nitrogen fixation. Conversely, there may be a negative impact on the inoculated plants, demonstrated through decreased survival and reduced root collar diameter growth after transplantation.

A greenhouse study by Laws and Graves (2005), found *Alnus maritima* seedlings grown with *Frankia* in soil without nitrogen addition formed the most nodules, but the seedlings had irregular shaped shoots and reduced greenness in the leaves. Reduced greenness in the leaves and irregular shaped shoots could be attributed to a diversion of carbon to high sink organs, such as root symbionts, instead of allocating carbon to leaves and stems for continued necessary maintenance and growth (Geiger and Servaites 1991). Our results suggest that after planting, inoculated plants divert carbon allocation to roots instead of leaves, indicated by the lack of change in total leaf carbon between Day 7 and Day 32 in comparison to control plants (Figure 7D). Total carbon values increased by 2% in control plants, and no control plants died in the first 50 days after planting. In contrast, total carbon of inoculated plants did not change, but survivorship decreased by approximately 10%. This may be due to a large carbon sink from increased carbon consumption by symbionts such as *Frankia* and mycorrhizal fungi (Tjepkema et al. 1986; Smith and Read 2008). It has been estimated that approximately 30% of total carbon produced is allocated to root symbionts, *Frankia* and fungi, with 15% allotted to each (Kennedy et al. 2015). *Alnus* is known for forming isolated symbiosis networks, meaning fungi and *Frankia* are not interconnected with other surrounding tree species (Kennedy et al. 2015). This could result in a disadvantage for the seedling, bacteria and ECM fungus due to

the seedling being the only carbon source. Photosynthetic rates in *Alnus* are comparable to other non-nitrogen fixing broad leaf tree species, but there is a higher demand on *Alnus* plants for carbon because of their root symbionts (Agren and Ingestad 1987; Koike 1990; Kennedy et al. 2015). In addition, *A. maritima* has higher light requirements than other alders for growth and maintenance (Schrader et al. 2006).

Natural colonization of control plants by naturally occurring soil microbes did occur in the field, and similar to previous studies of other alders, formation of nodules and the fixation of nitrogen typically occurred around 2-3 weeks depending upon the species and environmental conditions (Huss-Danell 1978; Wall and Huss-Danell 1997). Because of reduced survivorship of seedlings inoculated with the nodule microflora prior to planting and the occurrence of natural inoculation, we conclude that for management purposes pre-inoculation with nodule suspension is not necessary for successful transplantation of *A. maritima* seedlings. In nature, the mortality of young seedlings is potentially due to the physiological stress imposed by the microbiological community for carbohydrates when seedling nitrogen and phosphorus demand is low.

Conservation. Successful plant conservation efforts require information about the critical life stages of seed and seedling survival and establishment. Understanding how germination and dormancy impact *A. maritima*'s ability to re-establish is critical to maintaining the existing populations and potentially establishing new ones

(Schrader and Graves 2000b; Jones and Gibson 2012). With no genetically new individuals establishing through natural processes in the few, small, remaining populations, we recommend out-planting of older *A. maritima* seedlings or saplings to increase the chance of establishment and minimize the potential for extinction of the Oklahoma subspecies. Seedlings or saplings should be planted in areas with limited competition for light resources from later successional species. If establishment from seeds is the objective, then other aspects of *A. maritima*'s ecology needs to be considered. Rice and Gibson (2009) observed that seedlings that had germinated in the field ultimately died because they were growing in shaded conditions, and Schrader et al. (2006) demonstrated that seedlings and adults have extremely low shade tolerance. Their findings combined with our results indicate that management strategies hoping to establish new individuals from seed should at least remove the canopy, reduce the presence of competitors, and create exposed, high light conditions required for *A. maritima* in its native riparian environments.

The role of the soil microbiome in seaside alder conservation will require further study. If *Frankia* and its ectomycorrhizal species are a detrimental resource sink to seedlings, then their role in seedling establishment should be considered in any management plan. If soils where *A. maritima* currently grows have high *Frankia* populations, then sowing seeds in locations with existing seaside alders may not be a viable management option. As far as we know, the demand for carbon from the microbiological community associated with *A. maritima* seedlings has not been evaluated. A controlled study investigating the potential cost of inoculation with

Frankia and ECM fungi at early seedling stages (<9 months old) under different shading and nutrient conditions would indicate if seedlings are able to allocate carbon for growth and maintenance as well as support its symbionts. As pressures from climate change and the growing anthropological demands on the *A. maritima* habitat threaten the current adult populations existence, additional knowledge about its fundamental seed and seedling biology will be essential for conservation management plans.

Acknowledgments

The authors thank: M. Kistenmacher, Jona Tucker, Oklahoma Department of Wildlife Conservation, and Boy Scouts of America Camp Simpson. This was funded in part by the Nature Conservancy.

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Tables

Table 1. Average change \pm standard deviation in root collar diameter and height for seedlings grown in control, clipped and burned plots without (0-225 days) competitor and with (225-440) competitor.

Treatment	Average Δ Root Collar Diameter (mm)		Average Δ Height (cm)	
	0-225 (days)	225-440 (days)	0-225 (days)	225-440 (days)
Control	4 \pm 1.21	10.41 \pm 3.12	48.7 \pm 12.35	41.33 \pm 11.9
Clipped	3.9 \pm 1.5	7.07 \pm 2.93	48.25 \pm 12.84	33.2 \pm 14.9
Burned	2.23 \pm 1.9	5.6 \pm 1.31	50.61 \pm 14.8	45.2 \pm 13.8

Figures

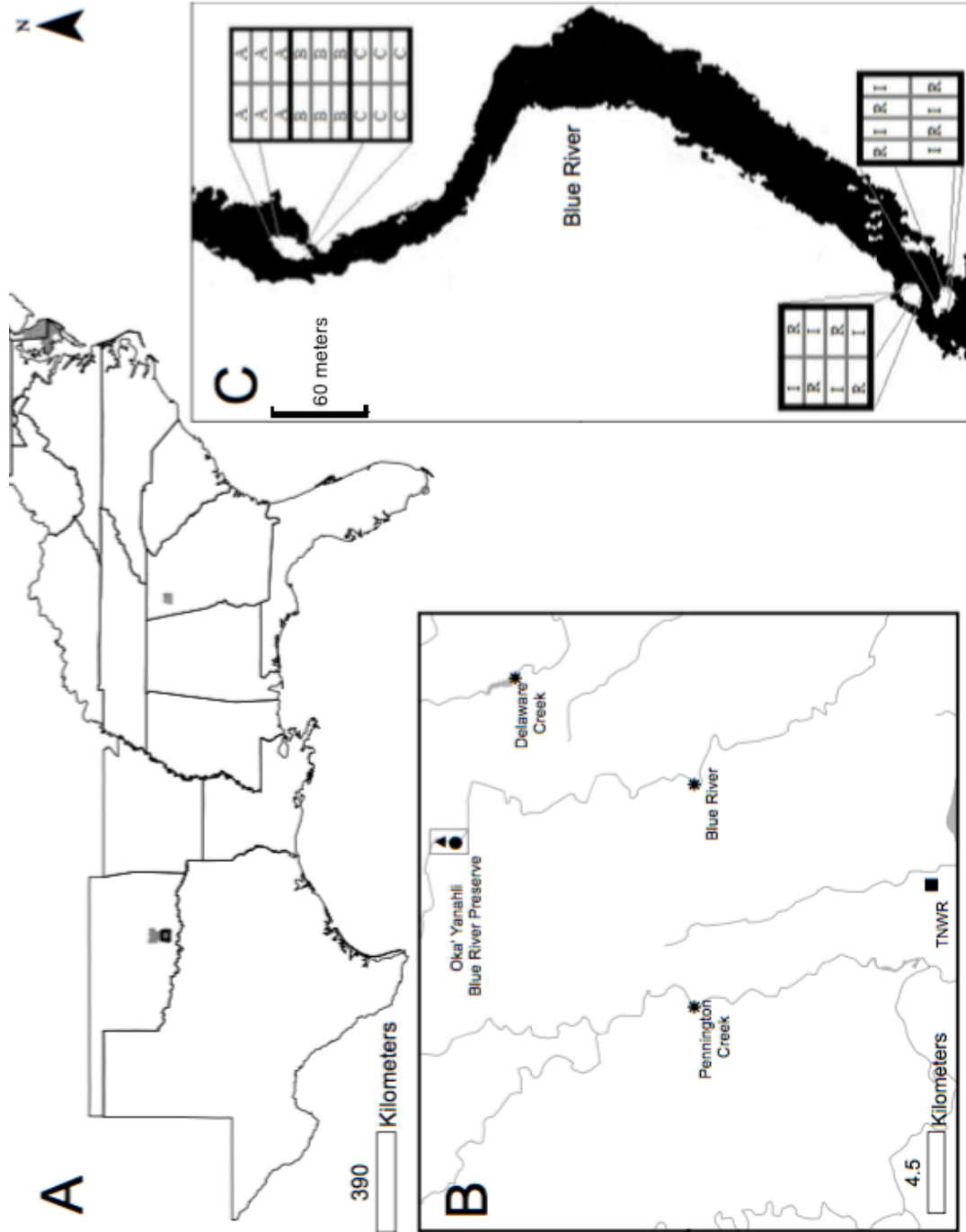


Figure 1. (A) *Alnus maritima* regional populations with Oklahoma, Georgia, Delaware and Maryland counties that contain individuals shaded grey. Black box indicating inset map B. (B) Seed collection sites at three extant *Alnus maritima* populations (*) along the Blue River, Delaware Creek and Pennington Creek in Oklahoma. Tishomingo National Wildlife Refuge (TNWR) is the location where seeds were buried for stratification. Oka' Yanahli Blue River Preserve, represented by triangle and circle, is the location of seedling establishment experiments. Box surrounding Oka' Yanahli Blue River Preserve indicates inset map C. (C) Oka' Yanahli Blue River Preserve has three islands where seedling establishment experiments were conducted. Representation of treatment plots with the northern island containing the disturbance experiment (control(A), clipped (B), and burned (C)). The two southern islands had control (R) and inoculated (I) seedlings used in the microbiome study.

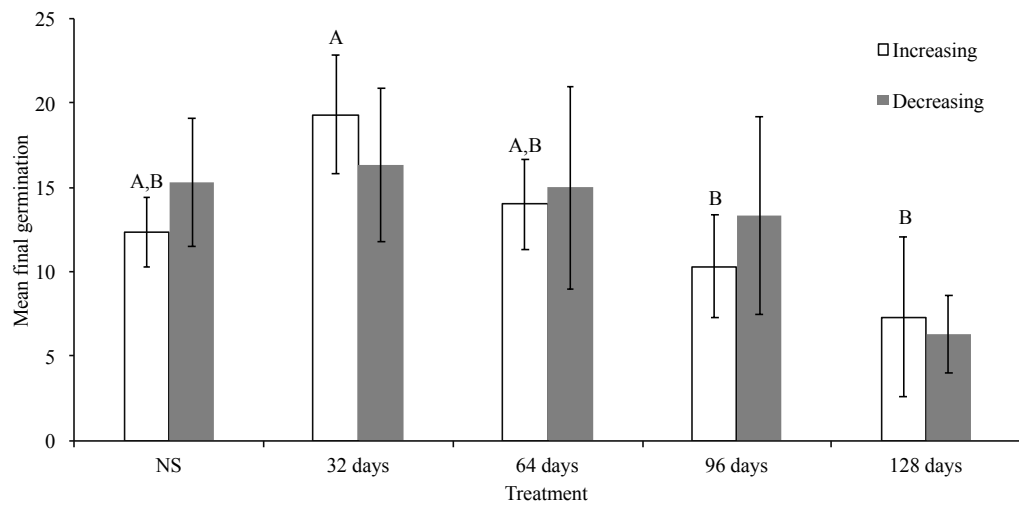


Figure 2. Mean final number of seed germination out of 50 seeds per replicate for non-stratified (NS), 32, 64, 96 and 128 day stratified seeds under increasing temperature regime (non-shaded bars) and decreasing temperature regime (shaded bars). Error Bars represent one standard deviation. Letters above columns distinguish significant differences ($p < 0.05$) between treatments for increasing temperature regime (non-shaded bars). No significant difference was found for decreasing temperature regime (shaded bars).

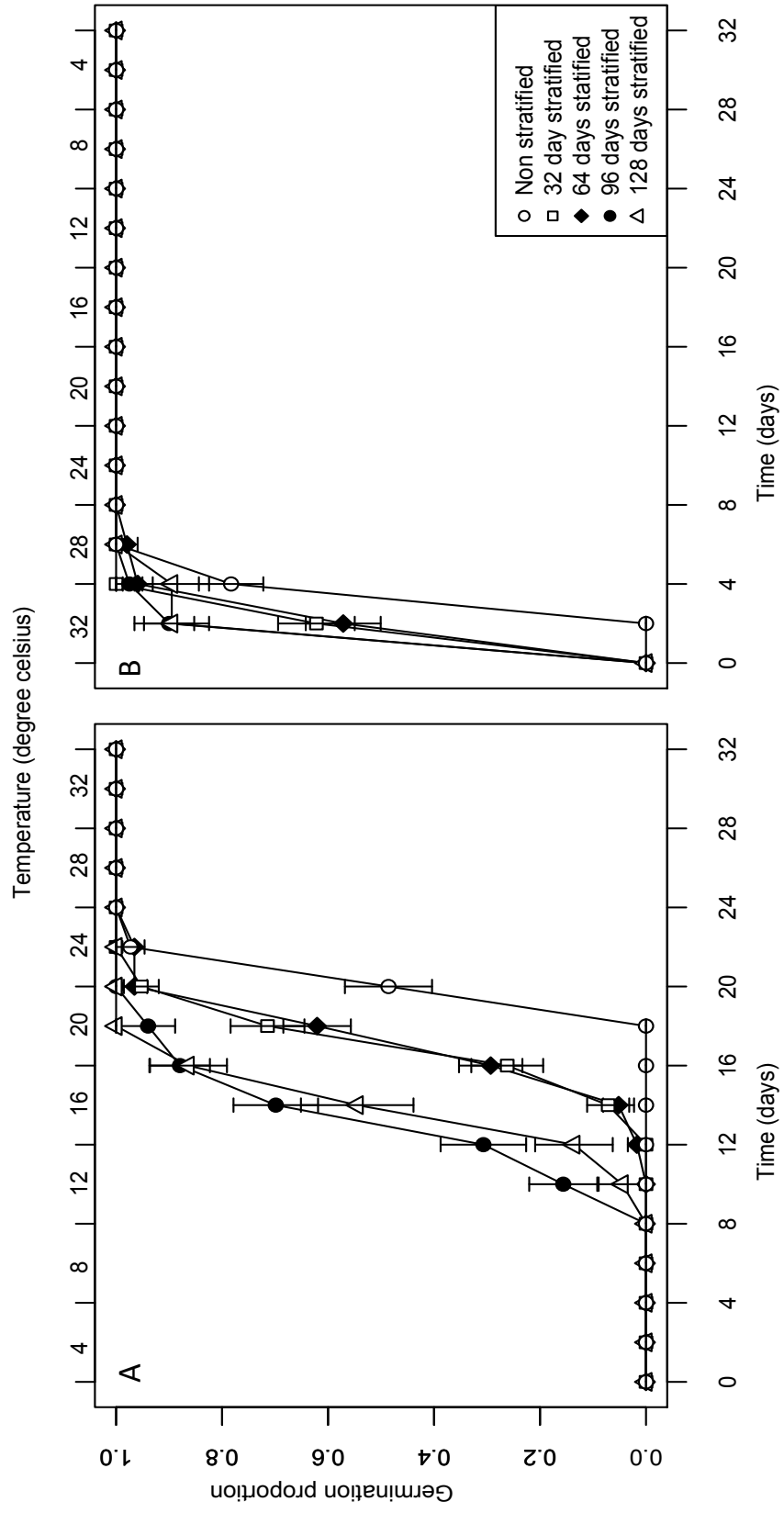


Figure 3. Kaplan Meier Curve Analysis depicting germination proportion under increasing (A) or decreasing temperature regime (B) over time (days) with a secondary x-axis illustrating the change in temperature ($^{\circ}\text{C}$). Points represent different storage treatments (Non-stratified, 32 days, 64 days, 96 days and 128 days). Error bars represent standard error.

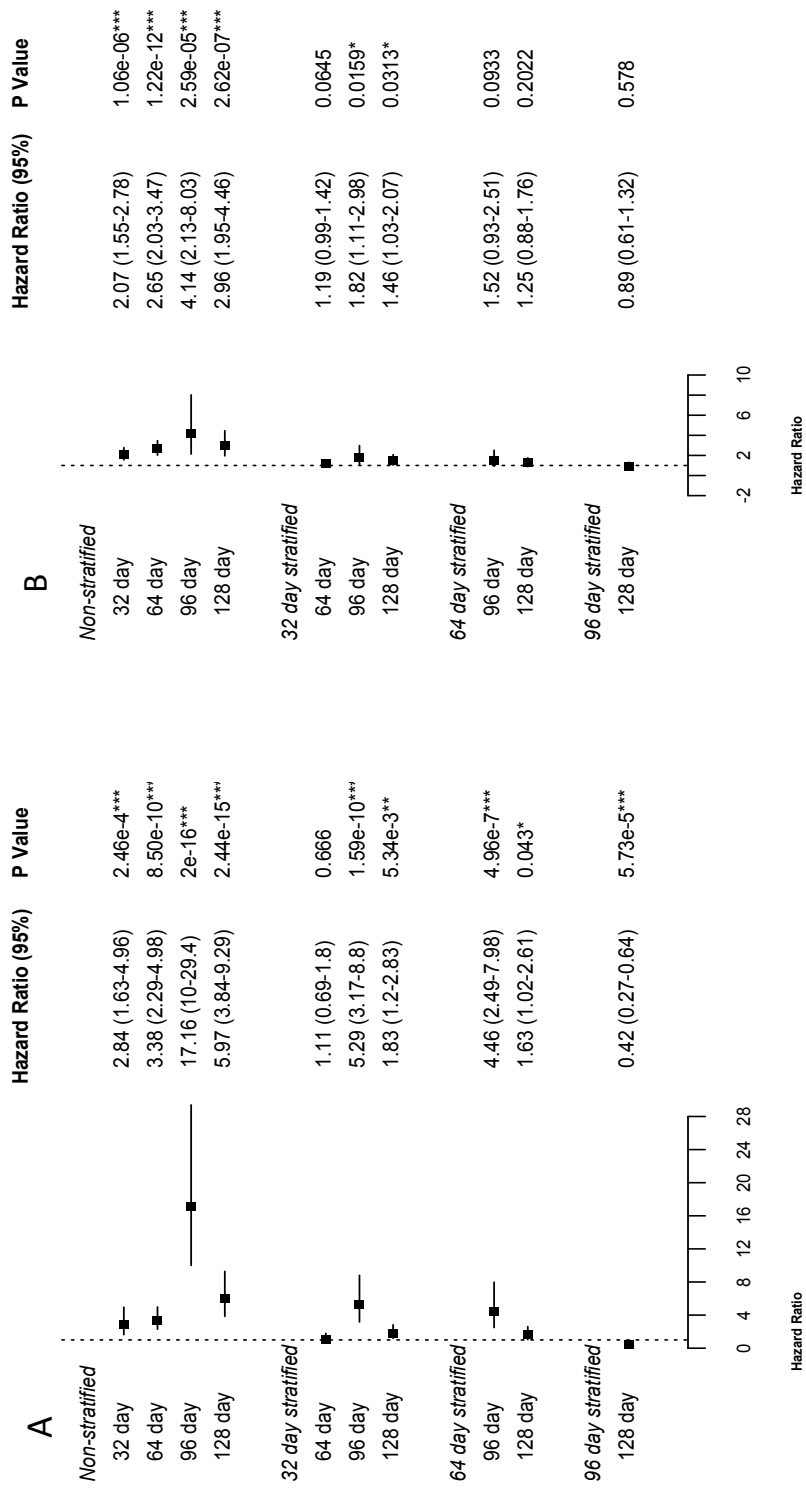


Figure 4. Hazard ratios (filled square) from Cox Proportional Hazards Model for storage treatments (32D, 64D, 96D, 128D) compared to baseline treatment (*italics*) under increasing temperature regime (A) and decreasing temperature regime (B). Bars represent 95 % confidence intervals. Dotted vertical line represents baseline hazard rate (i.e. hazard rate=1). Significance indicated by * with $p=0.05$ (*), $p=0.05-0.001$ (**) and $p<0.001$ (***)).

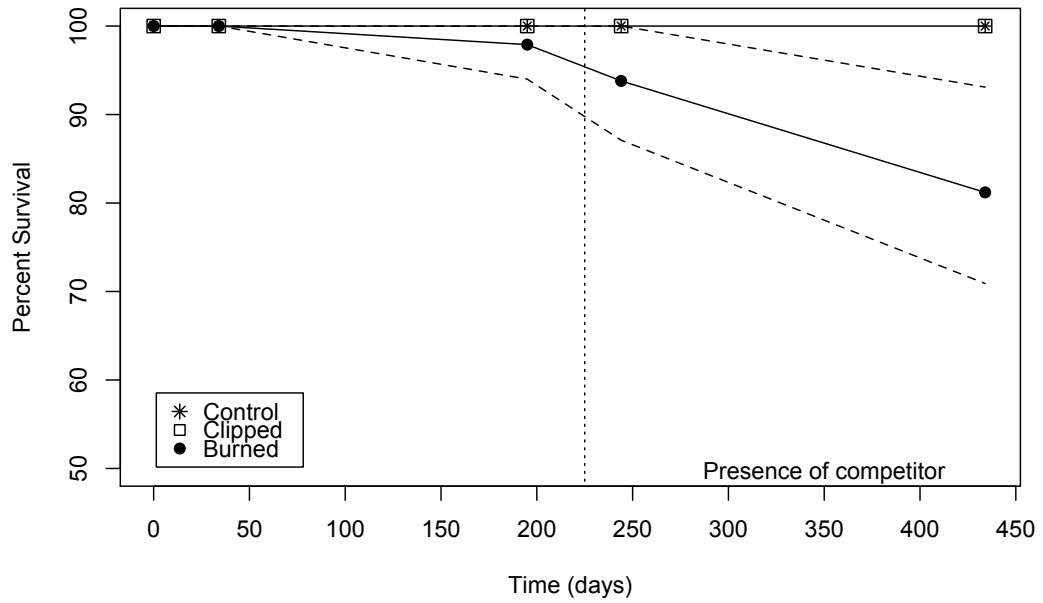


Figure 5. Kaplan-Meier Curve Analysis showing percent survival of seedlings planted in control, clipped and burned plots with time (days) since planting. Dashed line represents standard error. The vertical dotted line illustrates presence of *Strophostyles helvola* in the burn treatment.

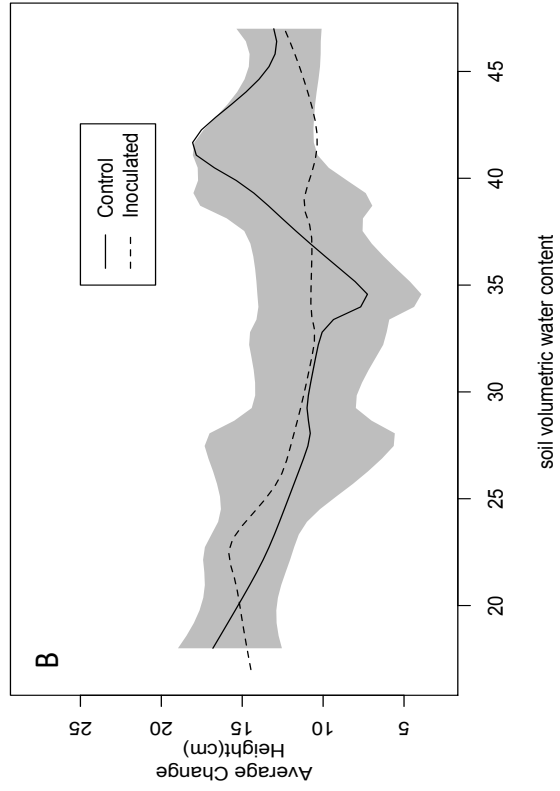
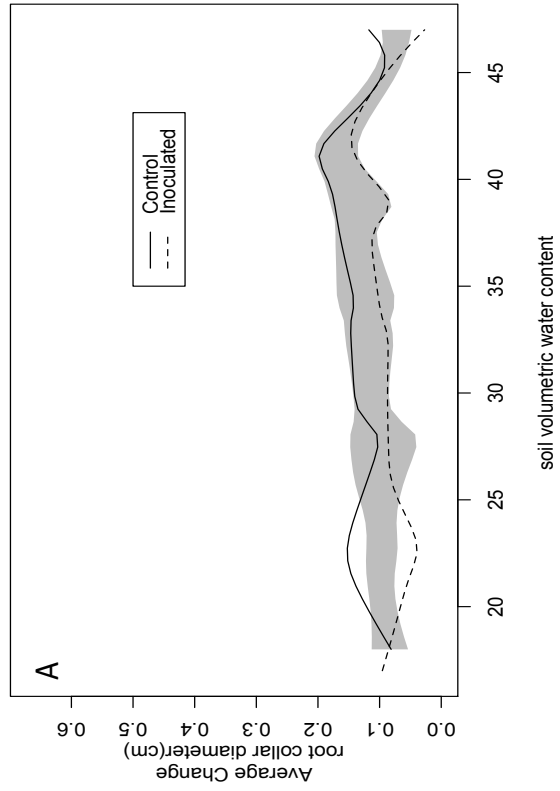


Figure 6. Average change in root collar diameter (A) and height (B) for control (solid line) and inoculated (dotted line) seedlings under different soil volumetric water content conditions. Shaded region illustrates the null hypothesis of equality for the nonparametric ANCOVA.

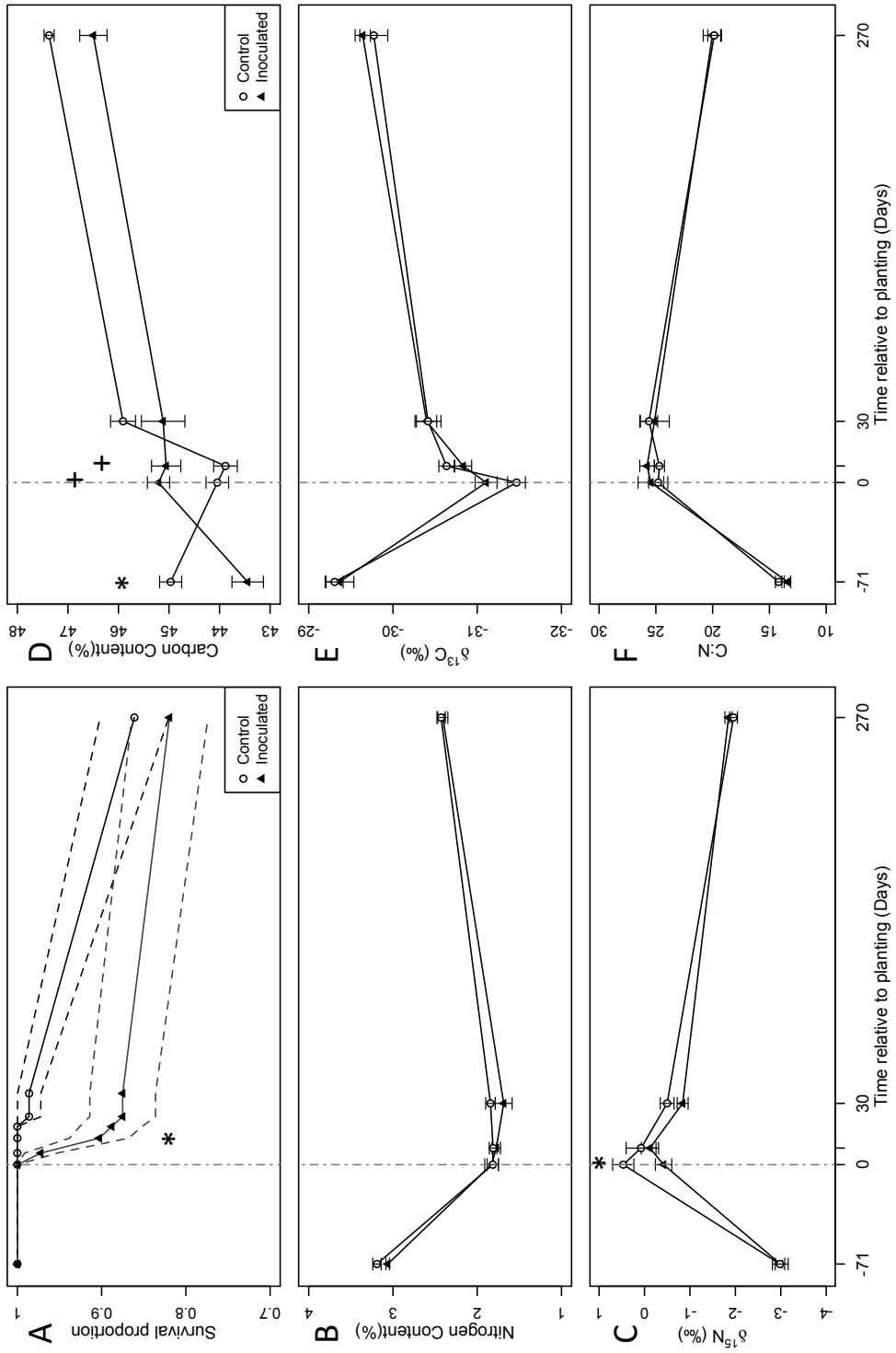


Figure 7. The change in (A) seedling survival proportion over time (days), (B) foliar nitrogen content (%), (C) foliar N15 isotope ($\delta^{15}\text{N}$), (D) foliar carbon content (%), (E) foliar C13 isotope ($\delta^{13}\text{C}$), and (F) C: N ratio for control (open circle) and inoculated individuals (shaded triangle) before and after planting (represented by dotted horizontal line). Error bars represent standard error. Asterisk (*) represents significance ($p < 0.05$) and the cross (+) represents near significance ($p < 0.08$).

Chapter 4- Summary

Climate change and the growing anthropogenic demands on the *Alnus maritima* habitat increase extinction pressures on current adult populations. Therefore, knowledge about the species biology is essential for conservation management plans. This study has found approximately 4,800 individuals along Oklahoma tributaries. Based on census data collected thus far, I hypothesize that there are an additional 1,000 individuals unaccounted for in the population. Although most of the areas surveyed were public conservation areas, conservationists should monitor population health and continue surveys of private land when accessible. However, if population size decreases, understanding how to re-establish seedlings will be a necessity for successful conservation management. This thesis provides insight into potential locations for seaside alder restoration and research projects that could be useful for understanding seedling re-establishment.

The unique distribution of *A. maritima* is hypothesized to be the remnants of a once larger distribution that has receded to the remaining regional populations (Stibolt 1981, Gibson et al 2008). *Alnus maritima* habitat requirements appear to be very broad because of the differences in conditions between the three regional populations and the hypothesized larger distribution (Stibolt 1981, Schrader and Graves 2002, Gibson et al. 2008). However, the two MaxEnt models provided in Chapter 2 suggest a restricted distribution in Oklahoma. For surveying and restoration projects, conservationists should focus on locations identified as having a high habitat suitability value for both the state and local models. If active management via

planting of seedlings is required in Oklahoma, conservationists should target areas of high habitat suitability within Pontotoc and Johnston counties. In addition, the methods from the models can be implemented for the Georgia and Delmarva populations to predict survey locations and potential areas for re-establishment projects.

Because few seedlings are found living in nature (Rice and Gibson 2009), active management is recommended and will be necessary to increase the number of *A. maritima* individuals. There are two topics where research is needed for *A. maritima* active management plans. First, seeds may be a suitable option for active management because seeds are not limiting the establishment of seaside alder, as demonstrated in chapter 3. However, little information is known about the survival of freshly germinated seedlings. Therefore, future studies should evaluate optimal seed sowing techniques and monitor young seedlings for survival.

Second, the third chapter shows that carbon demands were increased in the presence of a natural microbiological community which resulted in decreased growth and survival in 9-month old seedlings. As far as I know, the demand for carbon from the microbiological community associated with *A. maritima* seedlings has not been evaluated. However, recent studies in some legume species indicate that their microbiological community may be parasitic, exploiting this symbiotic relationship (Remigi et al. 2016). Therefore, future studies should investigate the potential cost of inoculation with *Frankia* and ECM fungi at early seedling stages (<9 months old).

This would indicate if seedlings can adequately allocate carbon for growth and maintenance as well as to its symbionts. Currently, young seedlings are found growing under large adult trees where exposure to *Frankia* and shaded conditions will likely occur (personnel observation). Therefore, the interaction between growth conditions (i.e. shading) coupled with the presence of the microbiological community should be carefully evaluated.

Few *Alnus maritima* subsp. *oklahomensis* adult individuals are being lost from the current populations, but without the addition of new individuals, the eventual loss will result in a decrease genetic diversity and ultimately population extinction. This threat is increased due to uncertainty associated with a changing climate and habitat quality. Therefore, I think that out-planting of *A. maritima* seedlings should be the primary goal of conservationists. Out-planting will not only increase the possibility of this species survival but will help preserve the riparian forest habitat that is also under pressure from growing anthropogenic demands.

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