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Germination, Survival, and Establishment of a Rare Riparian Species *Alnus maritima*

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ABSTRACT

Seed mortality due to low winter temperatures has been proposed as an explanation for the lack of seedling recruitment in natural populations of the rare riparian species *Alnus maritima*, but other factors such as the absence of essential root symbionts or canopy clearing disturbances could also limit establishment of new individuals. We investigated whether any of these factors could be identified as preventing recruitment into existing seaside alder populations. Stratification studies showed that not only can seeds withstand low temperatures, longer periods of cold stratification promote earlier seed germination and expand the temperature range for germination. Root microbiome studies unexpectedly found that seedlings inoculated with the native microbiome prior to planting had lower survival compared to uninoculated individuals, and uninoculated individuals declined in survivorship after natural inoculation in the field. Canopy disturbance by burning or clipping vegetation promoted neither seedling growth nor survival initially, with seedling survival lower in burned plots due to the release of an aggressively growing competitor. Our results show that physiological stress by microbial symbionts and competition with other species are likely primary limiting factors—more so than seed mortality from low temperatures—and should be the focus of future conservation efforts.
Key words: Betulaceae, conditional dormancy, disjunct distribution, nodule, stratification

INTRODUCTION

Seaside alder (*Alnus maritima* Muhl. ex Nutt., Betulaceae) is an extremely rare riparian species. It grows in three highly disjunct regional populations (the Delmarva Peninsula of Delaware, Maryland, and Virgina; northwest Georgia; and southcentral Oklahoma; Figure 1A) that are remnants of a once larger distribution that likely retracted following the Pleistocene glacial period (Little 1971, Furlow 1979, Stibolt 1981, Schrader and Graves 2000a, 2002, 2004, Gibson et al. 2008, Jones and Gibson 2011). Seaside alder has a G3 global conservation status, indicating it is threatened and faces risk of extinction throughout its range (Shaw et al. 2014), but the regional rankings vary among subspecies. *A. maritima* subsp. *maritima* is scattered in clusters along the Nanticoke River, Marshyhope Creek, and several lakes and ponds in the Delmarva Peninsula, and has a state conservation ranking of S3, indicating moderate extinction risk (Harrison 2016). The Georgia subspecies, *A. maritima* subsp. *georgiensis*, occurs in a single critically imperiled population in Drummond Swamp, located in Bartow County in northwest Georgia. This population has an S1 ranking, indicating it faces very high risk of extinction (World Conservation Monitoring Centre 1998, Chafin 2007). In Oklahoma, *A. maritima* subsp. *oklahomensis* grows in several small, isolated populations located along spring fed tributaries of the Blue River, Pennington Creek, and Delaware Creek in Johnston and Ponotoc Counties. The Oklahoma subspecies also has an S1 state conservation status, indicating high risk of extinction (Shaw et al. 2014).

Despite trees producing numerous viable seeds annually, there is no known establishment of new individuals from seed in any seaside alder population (Rice and Gibson 2009). Woody plants

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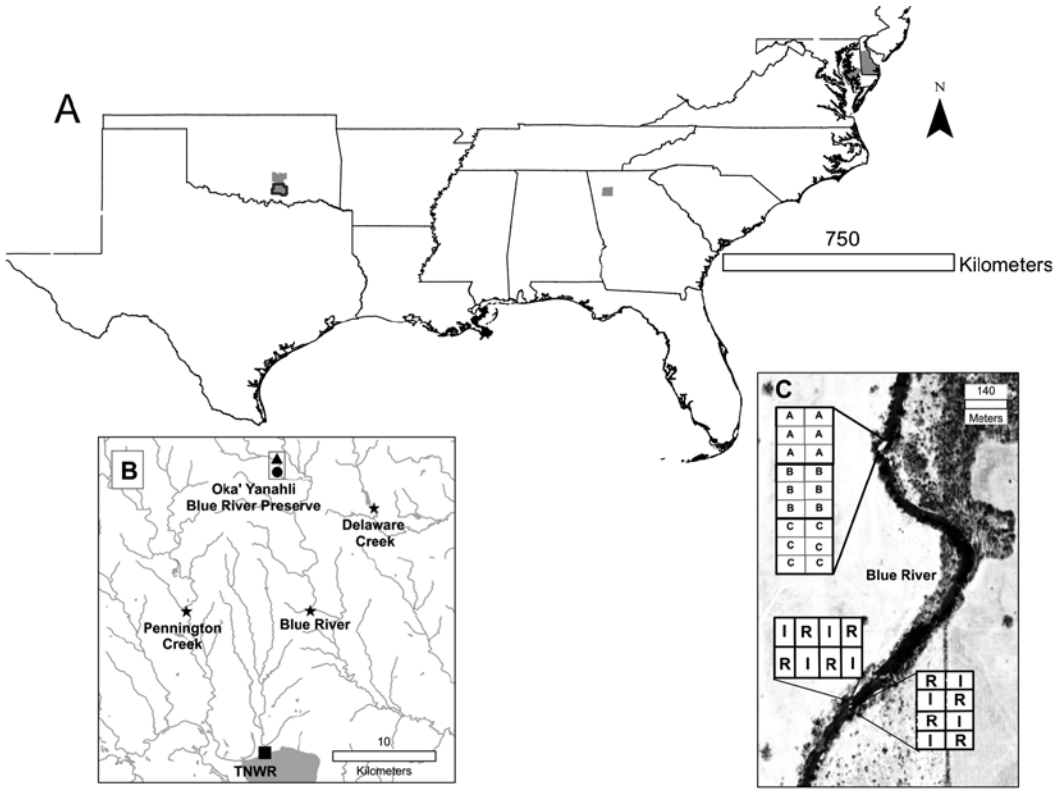

Figure 1. A. *Alnus maritima* regional populations with Oklahoma, Georgia, Delaware and Maryland counties that contain individuals shaded grey. Black box indicates area of inset map B. **B.** Seed collection sites at three extant *A. maritima* populations (★) along the Blue River, Delaware Creek and 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 ▲ and ●, is the location of seedling establishment experiments. Box surrounding Oka' Yanahli Blue River Preserve indicates area of inset map C. **C.** Oka' Yanahli Blue River Preserve has three islands where seedling establishment experiments were conducted. Representation of treatment plots on 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.

that experience frequent disturbance often have reduced regeneration from seed and result to resprouting as a life history strategy (Bellingham and Sparrow 2000). 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 population extinction as adults die and are not replaced. Successful conservation and management of rare plant species in instances such as this require basic knowledge of seed 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 (Poorter 2007), we conducted three studies of seed and seedling ecology to identify potential reasons for the absence of new individuals establishing from seed in this unique, riparian tree.

Schrader and Graves (2000b) proposed that low winter temperatures kill developing and mature seeds, thereby reducing seedling establishment in *A. maritima*. However, *A. maritima* may be similar to other alders and have either non-dormant or conditionally dormant seeds that would actually

benefit from exposure to cold temperatures (Baskin and Baskin 2014). Exposure to moist soil and temperatures between 0°C and 10°C can cold-stratify seeds and alter their physiological features causing either a shift from dormant to non-dormant status or broaden the range of threshold conditions that initiate germination (Baskin and Baskin 2004, 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 2004, Baskin and Baskin 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).

In addition to seed viability, interactions with other members of the community can influence seedling establishment. *Alnus* species frequently form symbiotic relationships in their root microbiome with arbuscular ectomycorrhizae (Molina et al. 1994, 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) that provide alders with essential limiting nutrients, thereby increasing plant growth and competitive ability compared to individuals without the root symbionts (Arnebrant et al. 1993, Hurd et al. 2001, Yamanaka et al. 2003, So et al. 2011, Bissonnette et al. 2014, Kennedy et al. 2015). Following germination, seedling establishment is also shaped by the abiotic environment and competitive interactions with other species in the community (Helenurm 1998, Kolar and Lodge 2001). Like other alders, *A. maritima* is an early to mid-successional, riparian species. Because human activities have altered natural disturbance regimes in riparian environments, there are fewer open habitats with high water and light necessary for establishment. Likewise, there is greater competition with later successional species (Folke et al. 2004, Schrader et al. 2006, Rice and Gibson 2009). Loss of natural disturbance regimes and increased competition with later successional species can decrease survival of rare species in riparian systems and may be inhibiting establishment of seaside alder seedlings (Richardson et al. 2007, Arkle and Pilloid 2009, Godefroid et al. 2011).

We conducted a series of field-based experiments to investigate **1)** the influence of the seed bank thermal environment on seed dormancy and germination, **2)** the effect of symbionts in the soil microbiome on seedling survival, and **3)** the impact of disturbance on *A. maritima* seedling establishment. Our first objective was to clarify how cold stratification affects seed mortality, dormancy, and germination in seaside alder under *in situ* seedbank conditions. If Schrader and Graves' (2000b) hypothesis that exposure to low temperatures is causing high seed mortality and preventing establishment is correct, then seeds overwintering under natural soil conditions would 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 tested in an incubator than fresh seed (Baskin and Baskin 1988, 2014, Benech-Arnold et al. 2000, Allen et al. 2007). Our second objective 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. Recruitment of *Alnus* species may be difficult in areas where root symbiont densities are low (Seeds and Bishop 2009). However, the costs to maintain root microflora in seedlings can also result in resource allocation constraints (Herms and Mattson 1992, Ballhorn et al. 2017). We predicted that seedlings inoculated with a nodule suspension prior to planting would have increased growth and survival compared to uninoculated individuals. Our last objective investigated the role of disturbance in seedling establishment by comparing 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 was expected to result in greater growth and higher survival of seedlings than in undisturbed conditions. The results from these three studies will indicate if the lack of new individuals into existing *A. maritima* populations is due to **(1)** seeds inability to survive winter stratification, **(2)** lack of symbionts resulting in low recruitment or **(3)** seedling inability to compete for adequate resources.

MATERIALS AND METHODS

Seed Dormancy and Germination

Similar to other alders, *Alnus 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 flowering in the fall while leaves are still present. After fertilization, seeds are retained in catkins where they overwinter until they are released the following fall. This is dramatically dissimilar from the more common alder species in subgenus *Alnus* that 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 ripe seeds for germination studies in October 2012 at Pennington Creek Crossing (34°19'20.2"N, 96°42'21.4"W), Boy Scouts of America Camp Simpson on Delaware Creek (34°24'38.6"N, 96°32'35.2"W), and United States Fish and Wildlife Service Blue River Public Fishing and Hunting Area (34°19'25.4"N, 96°35'36.7"W) in Johnston County, OK (Figure 1B). Mature but unopened catkins were collected from 20 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 × 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-day stored seeds (designated 128D), and 160-day stored seeds (designated 160D). Seeds were buried at a depth of 20 cm at Tishomingo National Wildlife Refuge (TNWR), 15 miles south of the known *Alnus maritima* locations (Figure 1B). TNWR has a mean annual precipitation of 98.7 cm and average 30 cm soil temperatures of 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).

Germination trials were conducted under temperature regimes mimicking increasing spring temperatures to identify the low temperature germination threshold and decreasing fall temperatures to evaluate the high temperature threshold and thereby evaluate seed dormancy states (Baskin and Baskin 2014). Differences in germination thresholds between non-stratified and stratified seeds 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 four days until reaching the maximum temperature of 32°C. Conversely, the decreasing temperature regime started at 32°C and decreased 4°C every four days until reaching 4°C (Washitani 1987, Batla and Benich Arnold 2003). 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 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 two 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 on previous tetrazolium tests that revealed brown solid or white nonsolid embryos were not viable (Schrader and Graves 2000a, 2000b, Baskin and Baskin 2014, J.P. Gibson and C. Ehardt-Kistenmacher, unpublished data). Germination trials using non-stratified seeds began on 1 November 2012. Seeds for stratification treatments were buried 2 November 2012, and a bag was retrieved from the storage location every 32 days to begin the next 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. We used an

Extended Cox Proportional Hazards Model with time independent heaviside functions in R (R Core Team 2014) to calculate Hazard Ratios (HR) as described by Kleinbaum and Klein (2012) and Kistenmacher and Gibson (2016). For this study, we calculated HR-values as the ratio between the germination rates of seeds in the shorter duration and longer duration stratification treatment to indicate the conditional probability of germination occurring during a given time interval due to a particular treatment. The Cox Proportional Hazard Model assumption of parallelism among the germination curves was checked using a Kaplan-Meier curve analysis function *Survfit* in the R package "Survival". If the model assumption for parallelism failed, then heaviside functions (*Hv*) were used to split data and compare HR between groups before and after the curves intersected (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 they were used in disturbance or microbiome experiments.

Root microbiome

We compared seedling establishment and growth between plants that had been inoculated with the native root microbiota and those that had not to evaluate how the bacteria and fungi associated with seaside alder roots influence seedling establishment. We raised 144 seedlings for nine months under greenhouse conditions at the University of Oklahoma. After 7.5 months, seedlings were randomly assigned to uninoculated reference (i.e., control) and inoculated 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 three–five 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, seven-month-old seedlings were transplanted from flats into 15.24 cm pots. During transplanting, plants in the inoculated treatment group received 10 mL 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 had similar size (approximately 14×14 m). Islands were cleared of herbaceous vegetation and divided into four 1.5×3 m plots per treatment (Figure 1C). In August 2013, nine seedlings were planted within each plot for a total of 36 seedlings per treatment on each island. Fencing was installed around the plots to exclude large herbivores. Height and root collar diameter were measured to the nearest hundredth at planting and in May 2014. Seedlings were monitored for mortality events at 7, 14, 21, 30, 40, 270 days after planting. We analyzed seedling leaf chemistry for percent nitrogen (%N) and abundance of foliar nitrogen isotopes ($\delta^{15}N$) to determine differences in nitrogen fixation between inoculated and control plants. Control plants' natural foliar $\delta^{15}N$ signature is different than that of inoculated plants, because the control plant nitrogen isotope signature will mimic predominantly nitrogen from the soil whereas the inoculated plant nitrogen isotope signature will contain soil nitrogen as well as nitrogen from nitrogen fixating bacteria (Kohl et al. 1980). Leaves for nitrogen and isotope analysis were collected before inoculation, one day before transplanting in the field, and at one week, four weeks and eight months after transplanting. At each collection date, we collected a leaf from the lower, middle and upper stem from 24 seedlings per treatment. To keep nitrogen values stable, leaf samples were stored on ice during transport from the field site to the lab. Leaves were then dried at 60°C for three days in a forced air oven (Shel Lab®) and then ground to a fine powder using a Retsch MM200® grinder. For each sample, approximately 2 mg of ground leaf material was weighed out using a Sartorius Microbalance (CPA2P®), and packaged in

a 4×6 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 0.3% for $\delta^{15}N$.

Canopy disturbance

We conducted disturbance experiments on a 14×28 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. Each zone contained six 4×4 m plots (Figure 1C). In August 2013, vegetation in both the clipped and burned plots was clipped but not removed. On 6 September 2013, the Nature Conservancy burned the southern part of the island. Scattering an additional hay bale over burn plots increased fuel load and fire intensity. After clipping and burning, a barrier fence was installed to prevent large herbivores from damaging the transplants. A total of 144, 10-month-old seedlings (average height 49.23±17.85 cm, average stem diameter of 0.90±0.51 cm) were planted on 15 September 2013. There were 48 seedling per treatment and 8 seedlings per plot. Seedlings were monitored for survival, root collar diameter, and height using digital calipers. At planting, the soil volumetric water content (SVWC) at 20 cm deep was measured using HydroSense II® CS658 rods (Campbell Scientific Logan, Utah) from the east and west ends of each plot to account for differences in growth attributed to water availability.

Seedling survival and growth data analysis

A Cox Proportional Hazards Model was used to calculate HR-values and determine if there was a difference in seedling survival between inoculated and uninoculated plants in the microbiome experiment or among treatments in the disturbance experiment. Because a native vine species, *Strophostyles helvola* (L.) Elliott, spread rapidly and dominated burned plots approximately 225 days after planting, data for the disturbance study were divided into two time periods, prior to *S. helvola* establishment in the burned site (0–225 days) and post (225–440 days) *S. helvola* establishment on the burned sites. Heaviside functions were then used to calculate HR-values 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) and $\delta^{15}N$ values, between inoculated and control plants. Because plants with and without nodules will be accessing different nitrogen sources, we anticipate differences in nitrogen chemistry between them. Leaf chemistry sample data were analyzed using the "nlme" package with multiple comparisons ("multcomp" package) with a Bonferroni correction in R.

RESULTS

Seed germination

Under the increasing temperature regime, the highest mean final seed germination occurred in the 32D stratification treatment (Figure 2). Germination declined as storage time increased, resulting in the lowest mean final germination in 128D seeds. Seeds in the 160D treatment could not be used in the analysis 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. For all treatments, a majority of germination occurred between 8°C to 24°C (Figure 3A). However, seeds stratified for longer periods of time began germinating at lower temperatures. The HR-values indicate that 96D seeds had significantly higher germination rates than all other treatments, followed by 128D seeds, (Figure 4A). Germination did not differ between 64D and 32D seeds, and all treatments had higher germination than NS seeds.

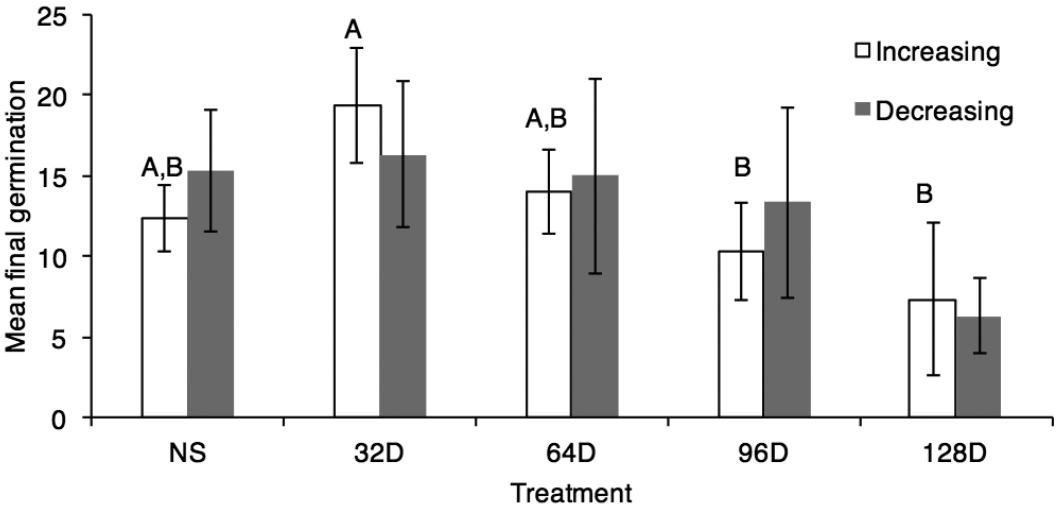

Figure 2. Mean final number of seed germination out of 50 seeds per replicate for NS, 32D, 64D, 96D and 128D stratified seeds under increasing temperature regime (non-shaded bars) and decreasing temperature regime (shaded bars). Error bars represent standard error Letters above columns distinguish significant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

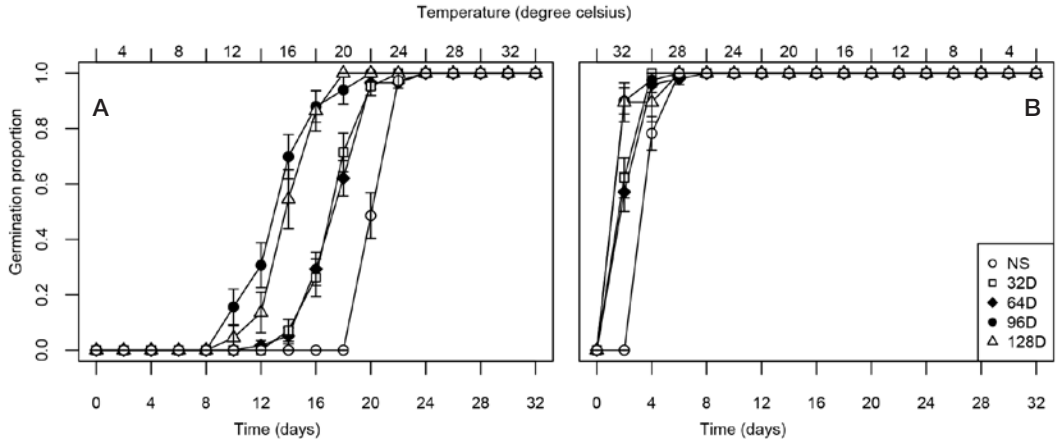

Figure 3. Kaplan Meier Curve Analysis depicting germination proportion under increasing (A) or decreasing temperature regime (B) over time (days) with a secondary x-axis illustrating the change in temperature (°C). Points represent different storage treatments (NS, 32D, 64D, 96D and 128D). Error bars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

Under the decreasing temperature regime, mean seed germination declined slightly with longer storage time (Figure 2), but there was no difference in the final seed germination among treatments (ANOVA, df=4, F=2.201, p=0.142). All germination occurred between 32°C and 28°C. Stratified seeds were more likely to initiate germination at 32°C than NS seeds and had significantly higher germination rates (Figures 3B and 4B). The only other significant differences in germination rates were 32D seeds having lower germination rates than the 96D and 128D.

Root microbiome

Although similar in diameter initially, control plants had a significantly greater increase in root collar diameter during the experiment than inoculated plants (non-parametric ANCOVA, p=0.0004,

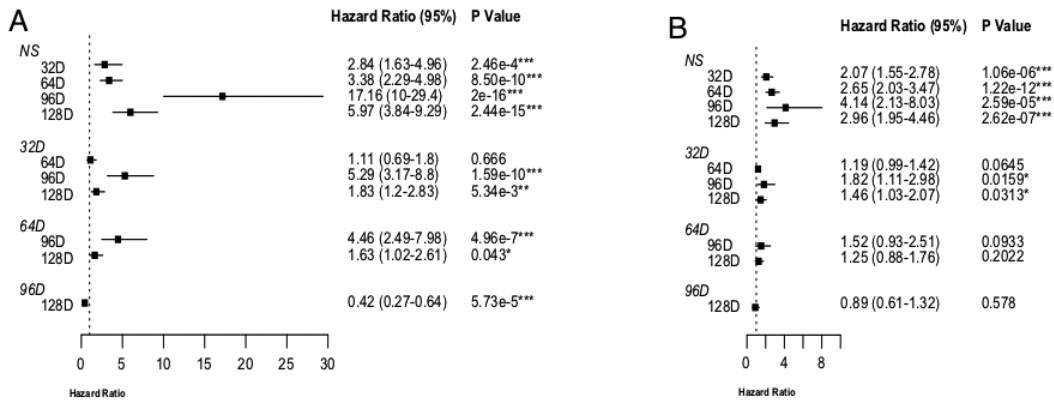

Figure 4. Hazard ratios (filled square) from Cox Proportional Hazards Model for storage treatments (32D, 64D, 96D, 128D) compared to baseline treatment (italics) under increasing temperature regime (A) and decreasing temperature regime (B). Bars represent 95% confidence intervals. Dotted vertical line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

Figure 5A). However, the average change in height was not different between inoculated and control plants (p=0.3802, Figure 5B). Within the first 30 days after planting, inoculated seedlings had significantly lower survival than control plants (HR=9.571, p=0.028, Figure 6A). However, 270 days after planting, there was no difference in survival rates between remaining 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 6B) throughout the experiment. Foliar δ^{15}N was the same between control and inoculated plants at inoculation (z=0.03, p=1), but δ^{15}N content was significantly higher in control plants than in inoculated plants (z=3.481, p=0.023) two months after inoculation indicating nitrogen in control plants is coming from the soil and not nitrogen fixation. Eventually, foliar δ^{15}N of control and inoculated plants declined slightly and ultimately converged after planting in the field.

Canopy disturbance

Seedlings from control, clipped and burned plots all showed high survival during the first 225 days after planting, with only one individual dying in the burn plot (Figure 7). There were also no differences in seedling root collar diameter (non-parametric ANCOVA, p=0.4242) or height (non-parametric ANCOVA, p=0.9423) between the control, clipped, or burned plots during this time period (Table 1). However, between days 225 and 440, *S. helvola* spread and began to dominate the burned plots. After that time, seedling survival in burned plots decreased to 80%, but all seedlings survived in control and clipped plots. Consequently, seedlings in burned plots 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). There were no differences in seedling root collar diameter increase (non-parametric ANCOVA, p=0.247) or height increase (non-parametric ANCOVA, p=0.7473) in the surviving seedlings between the burn, clipped or control plots (Table 1).

DISCUSSION

Our three studies investigating potential factors responsible for the lack of recruitment of new *Alnus maritima* individuals into existing populations indicate that it is not due to lack of viable seed, but instead is due to failure of seedlings to establish. Germination analysis of field stratified seeds indicated that *A. maritima* adults produce numerous, viable seeds, and found no evidence to indicate that seed mortality experienced during the winter is limiting recruitment. Seeds are viable after overwintering in the soil seed bank under normal conditions and show germination behavior typical of other temperate species that disperse seeds in the fall and germinate in the spring. Our

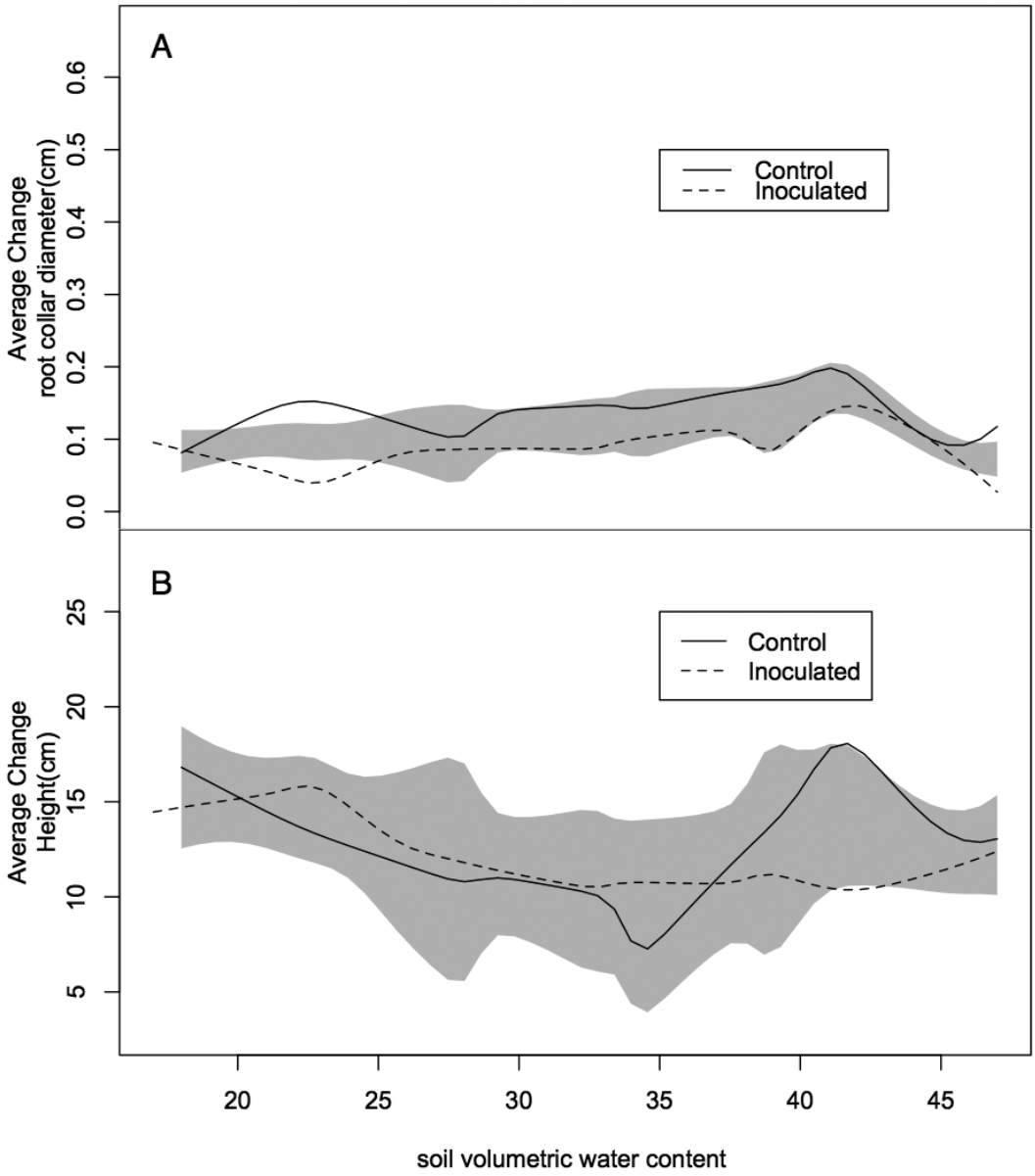

Figure 5. Average change in root collar diameter (A) and height (B) for control (solid line) and inoculated (dotted line) seedlings under different soil volumetric water content conditions. Shaded region illustrates the null hypothesis of equality for the nonparametric ANCOVA.

results suggest that absence of seed bed conditions and appropriate sites for seedling establishment during the early seedling life stage is likely the critical phase limiting the establishment of new individuals due to competition with other species, and potentially detrimental interactions between young seedlings and their root microbiome.

The *in situ* stratification studies did not detect significant seed bank mortality. Germination curves for *A. maritima* indicate high seed viability after winter soil storage and that seeds are capable of spring germination. Both fresh and field stratified seeds germinated readily and achieved

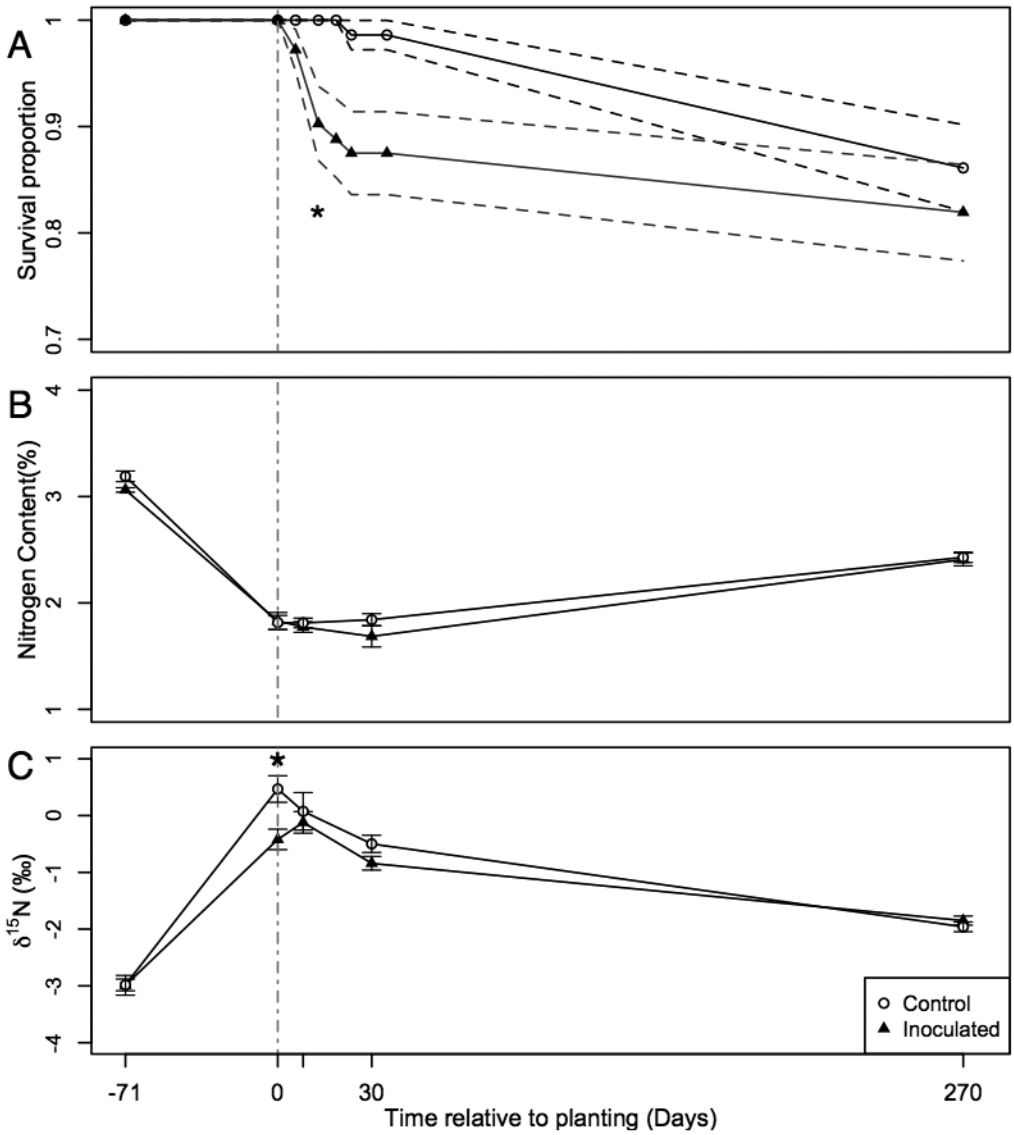

Figure 6. Seedling survival proportion (A), (B) foliar nitrogen content (%), (C) foliar N15 isotope (^{15}N) over time (days) for control (open circle) and inoculated individuals (shaded triangle). Dotted horizontal line indicates day of planting. Error bars represent standard error. Asterisk (*) represents treatment significance ($p<0.05$).

similarly high germination totals. Rather than reduce viability, stratification for 96 and 128 days promoted germination at lower temperatures in the increasing temperature regime and at higher temperatures in the decreasing temperature regime. Seeds stored for longer periods likewise showed high germination while *in situ* which further demonstrates the ability of *A. maritima* seeds to survive winter temperatures in the seed bank. Similar to other temperate species which disperse seeds in the fall, broadening of the low and high temperature thresholds for germination indicates *A. maritima* seeds possess non-deep conditional physiological dormancy (Baskin and Baskin 2014) in which exposure to cold winter temperatures promotes seed germination the following spring. Schrader and Graves (2000b), showed high seed mortality under extreme cold

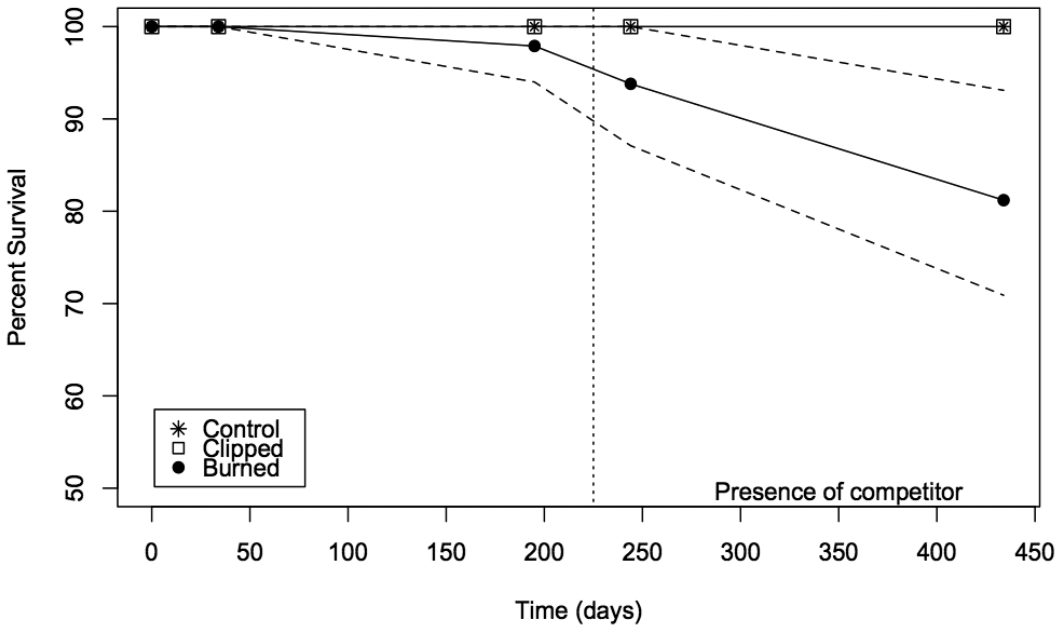

Figure 7. Kaplan-Meier Curve Analysis showing percent survival of seedlings planted in control, clipped and burned plots with time (days) since planting. Dashed line represents confidence interval. The vertical dotted line illustrates presence of *Strophostyles helvola* in the burn treatment.

Table 1. Average change ± standard error 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 for the burn treatment.

Treatment	Average Δ Root collar diameter (cm)		Average Δ Height (cm)	
	0–225 (Days)	225–440 (Days)	0–225 (Days)	225–440 (Days)
Control	0.4±0.12	1.04±0.31	48.7±12.35	41.33±11.9
Clipped	0.39±0.15	0.71±0.29	48.25±12.84	33.2±14.9
Burned	0.22±0.19	0.56±0.13	50.65±14.8	45.2±13.8

stratification conditions of three days at -15°C, a temperature that is approximately 10°C colder than south-central Oklahoma's average minimum air temperature (McPherson et al. 2007). Seeds in our study, however, experienced natural soil temperature and moisture conditions which can be 1–3°C warmer than air temperatures thereby providing an additional buffer against temperature extremes in the seed bank (McPherson et al. 2007, Fernández-Pascual et al. 2015). Our results are similar to a seed dormancy and germination study conducted on the non-threatened, well-adapted common alder (*A. glutinosa*) (Gosling et al. 2009) suggesting that seeds of *A. maritima* are not negatively affected by cold winter temperatures and therefore not limiting establishment.

Although many plant species experience higher establishment success after inoculation with root symbionts prior to planting (So et al. 2011), we found no benefit from pre-inoculation with a suspension of *Frankia* spp. and other members of the root microbiome prior to planting. Counter to expectations, seedlings that were inoculated with the microbial community prior to planting had a significantly lower chance of survival during the first 30 days than uninoculated plants. Inoculated plants also showed lower root collar growth. There was a difference in $\delta^{15}N$ isotope

signatures between inoculated plants and control plants at planting, suggesting that nitrogen was acquired from different sources (Shearer et al. 1978, Domenach et al. 1989, Kurdali et al. 1990, Boddey et al. 2000). However, after planting, nitrogen isotope signatures became more similar, which may be caused by uninoculated plants finally being colonized by the native soil microbiome. While we find no clear evidence of an effect on seedling nitrogen metabolism, there appears to be a negative effect on seedling survival due to the inoculation prior to planting. Laws and Graves (2005), showed that *Alnus maritima* seedlings grown in soil with *Frankia* and low nitrogen formed the most nodules, but the seedlings had irregular shaped shoots and reduced greenness in the leaves. This may be a consequence of high carbon consumption by *Frankia* and mycorrhizal fungi that diverts photosynthate to high sink organs, such as nodulating roots, instead of leaves and stems (Tjepkema et al. 1986, Geiger and Servaites 1991, Smith and Read 2008). Thus, while the nitrogen demand of young seedlings may be so low that inoculation provides no detectable benefit, there may be other demands by the microbiome on other resources, which inhibit successful seedling establishment. In a field study by Ballhorn et al. 2017, *A. rubra* Bong. (red alder) leaf carbohydrates were lower in *Frankia* inoculated seedlings, and seedlings had reduced height and increased susceptibility to herbivores suggesting resource allocation constraints. Further investigation is required to determine whether root symbionts are detrimentally influencing the allocation of nitrogen, carbon, and other resources between the seedlings and their root microbiome during nodulation, and how that influences seedling establishment and growth in *A. maritima* and other perennial woody species (Boddey et al. 2000). Studies in legumes have found that host may not be able to determine if symbiont strains are beneficial or harmful at initial colonization (Masson-Boivin and Sachs 2018) and depending on the interaction between rhizobia, host and abiotic conditions, the rhizobia may act as a parasite (Remigi et al. 2016).

Because seaside alder is highly shade intolerant (Schrader et all 2006), it is surprising that altering the above ground community to reduce shading and competition via clipping or burning did not promote significantly greater survival. Alders are often one of the first woody colonists after fire where exposed substrates and high light environments are optimal for establishment (Matthews 1992, Lantz et al. 2010). Seaside alder's sister species, *Alnus nepalensis*, is associated with colonization of burned sites (Swanson et al 2010, Tang et al 2013), but we found no evidence for enhanced growth in height and root collar diameter in surviving *A. maritima* individuals in burned or clipped plots. Seedlings in burned plots actually had reduced survival due to competition with a native, fast-growing, annual vine, *Strophostyles helvola*, whose seed germination was stimulated by fire, and the vines from those seed grew on and over the seaside alder seedlings leading to their mortality. While our results are show no clear benefit to seedling establishment by removing vegetation, there was clear evidence that disturbance from fire in Oklahoma is detrimental to seedling survival due the competitive biotic interactions it promoted.

Conservation

Successful conservation of species such as *Alnus maritima* requires identifying and providing the conditions necessary for seed and seedling survival and establishment. Understanding how germination and dormancy impact the ability to re-establish is critical to maintaining the existing populations and potentially establishing new ones (Schrader and Graves 2000b, Jones and Gibson 2012). Established trees produce ample seed annually and provide a large pool of propagules to establish new trees. If establishment from seeds is the objective, the role of the soil microbiome in seaside alder conservation will require further study. If *Frankia* and ectomycorrhizal species are initially 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. 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 within 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. Mortality of young seedlings in the field may potentially be due to the demand imposed by the microbiological community for carbohydrates when seedling nitrogen and phosphorus demand is low. Our results indicate that any inoculation with root microflora should not occur until seedlings are capable of withstanding the nutrient demands they place on the plant.

Because of its high light demands, *Alnus maritima* seedlings or saplings should be planted in areas with limited competition and shading. 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 by removing the canopy by creating high light conditions should be aware that this may likewise release competitors for *A. maritima*

Our studies have shown that low seedling and sapling survival in the Oklahoma populations are likely the predominant factors inhibiting establishment of new seaside alder individuals and not seed mortality. With no genetically new individuals establishing through natural processes in the few, small, remaining populations, we recommend out-planting of older *Alnus maritima* seedlings or saplings to increase the chance of establishment and minimize the loss of remaining genetic variation and protect against the detrimental impacts of genetic bottlenecks that can potentially contribute to the extinction of the Oklahoma subspecies. These factors should be investigated in Delmarva and Georgia populations to evaluate whether they are also the cause of limited establishment in those regions also.

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