ENVIRONMENTAL FACTORS AFFECTING WOODLAND LEGUME RESTORATION

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☐ Pass with no revisions ☐ Pass with minor revisions

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Abstract

Past efforts to reintroduce the native legume species *Desmodium glutinosum* and *Lespedeza violacea* into restored woodlands have not produced self-sustaining populations. Proposed factors preventing reintroduction include herbivory, persistent environmental effects of invasive shrubs, poor performance of commercial *Rhizobium* inoculants, and competitive displacement associated with elevated nitrogen availability. To address these factors, we conducted field experiments to determine how restoration maturity and six environmental conditions: light availability, soil moisture, soil pH, ammonium (NH_4), nitrate plus nitrite (NO_x), and phosphate (PO_4), affected survival and productivity of transplanted legume seedlings. Legume vegetative growth was not affected by environmental variables, but *D. glutinosum* survival was negatively correlated with soil moisture ($p = 0.034$) and NO_x ($p = 0.017$) and *L. violacea* fruit set increased with higher pH ($p = 0.023$) and more light ($p = 0.026$). Older restoration sites were correlated with lower NO_x ($p = 0.028$) and reduced light availability ($p < 0.009$). Controlled greenhouse experiments tested inoculant specificity and the effects of nitrogen addition and plant competition with neighboring grasses on seedling growth and productivity. Neither species-specific nor nonspecific commercial inoculants yielded viable root nodules. Competition did not affect *D. glutinosum* performance, while *L. violacea* aboveground biomass was reduced under competition with *Elymus villosus* ($p = 0.045$). High nitrogen addition caused reduced biomass in both species under all competition treatments, but this effect was likely due to direct toxicity of urea fertilizer. Data collected from these experiments will help develop protocol revisions and best practices for the reintroduction of woodland legumes in sites where previous restoration attempts have failed.

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Introduction

Roughly the size of the state of California, the 40 million hectare Central Forest-Grasslands Transition Zone marks the boundary between eastern deciduous forest and tallgrass prairie (Ricketts et al. 1999) and extends from the southern end of Lake Michigan in northern Illinois to northern Texas. Prior to widespread agriculture, this transitional biome, which surrounds Chicago and its neighboring suburbs, was dominated by a patchwork of oak woodland, savanna, and tallgrass prairie (Kline 2005a, Sauer 1998). Climate as well as fire and grazing disturbances shaped the region's plant communities in the past, but land-use change has profoundly altered these and other processes, resulting in loss of natural habitat area and species diversity, and susceptibility to invasion by aggressive species (King 1981, Leach and Givnish 1996, Levine 2000, MacDougall and Turkington 2005, Ramalho and Hobbs 2012).

As a result of prolonged fire suppression, the oak woodland areas of the Midwest have become heavily invaded by fire-intolerant woody species including *Rhamnus cathartica* (common buckthorn). An invasive shrub introduced from Europe in the early 1800s, buckthorn grows in dense stands throughout the Midwestern and Northeastern United States and parts of Canada (Wieseler 2005). It is shade and drought tolerant, and its fast growth and prodigious fruit production allow buckthorn to rapidly shade out native species (Klionsky et al. 2011, Knight et al. 2007). Buckthorn foliage is relatively high in nitrogen and decomposes quickly, thereby altering soil pH and nutrient cycling, resulting in loss of the litter layer and further suppression of fire through fuel elimination (Heneghan et al.

2006a, Wieseler 2005). Additionally, buckthorn-dominated areas have been shown to support higher densities of Eurasian earthworms (Heneghan et al. 2006b). Exotic earthworms accelerate decomposition rates and alter nutrient cycling and soil chemistry, establishing a positive feedback loop of mutually facilitated invasion with buckthorn (Nuzzo et al. 2009, Szlavecz et al. 2006). Mature buckthorn thickets exclude native plant species; soil under these thickets is bare and nitrogen enriched, and light penetration through the canopy is minimal. Long-lasting effects of invasion have resulted in fundamental changes to the plant communities and environmental conditions of restored woodlands (Bauer 2012, Bradshaw 1996, Foster et al. 2003).

As a result of fossil fuel combustion, use of industrial fertilizers, and large-scale farming of nitrogen-fixing crops, nitrogen has become much more abundant in the environment (Fenn et al. 2003b, Vitousek et al. 1997). Increased nitrogen availability disrupts nutrient cycling processes, increases eutrophication, promotes invasion by aggressive weedy species, and reduces biodiversity (Blumenthal et al. 2003, Clark and Tilman 2010, Gilliam 2006, Skogen et al. 2011). The resultant loss of native species has been particularly severe among species adapted to nitrogen-limited conditions such as legumes (Baer et al. 2004, Leach and Givnish 1996, Skogen et al. 2011, Vitousek et al. 1997). Legumes are typically poor competitors for light, and where abundant nitrogen promotes rapid growth of aggressive species, legume populations often decline (Suding et al. 2005, Wedin and Tilman 1996).

Research objectives

Two native woodland legume species, *Desmodium glutinosum* (Muhl. ex Willd.) Alph. Wood (pointed-leaf tick trefoil, henceforth *Desmodium*) and *Lespedeza violacea* (L.) Pers. (violet lespedeza, henceforth *Lespedeza*) have responded poorly to restoration efforts in McDonald Woods, an actively managed oak woodland in Glencoe, Illinois. Small populations of both species exist in woodland sites elsewhere in the region, but no natural recruitment of new plants has been observed at this site and extant populations are not increasing. While greenhouse-propagated seedlings have grown to reproductive maturity after transplantation, autumn seed distribution yields seedlings that do not reach maturity during the growing season (Steffen, personal communication). This evidence suggests that loss of young plants or poor habitat conditions is preventing legume population growth. Deer are known to preferentially browse on other species of the *Desmodium* and *Lespedeza* genera, so the large deer population in the area may pose a significant risk to young legumes in this woodland (Muir and Bow 2008, Skogen 2008). Lack of appropriate nitrogen-fixing bacteria, unsuitable environmental conditions resulting from legacy effects of buckthorn, loss of competitive advantage due to nitrogen deposition, and herbivory from overabundant deer have been proposed as possible explanations for failed legume restoration.

This project investigates factors potentially preventing viable reintroduction of these species in two parts. Chapter 1 reports a field experiment involving transplanted legume seedlings to answer the question: Under what environmental and restoration conditions are plant productivity and seedling survival improved or impaired? Chapter 2 reports a series

of greenhouse experiments conducted to answer two questions: Are inoculants commonly used in restoration appropriate for these species? And what are the effects of nitrogen deposition and resource competition on legume seedling productivity and survival? By combining field and greenhouse experiment, this study will assess the factors limiting legume performance in this habitat, which may guide future restoration efforts.

Chapter One: Field Experiments

Introduction

Although woodland restoration has produced improvements in species richness and habitat quality in McDonald Woods, *Desmodium* and *Lespedeza* have responded poorly to date. To test whether specific environmental conditions affect legume growth and survival, *Desmodium* and *Lespedeza* seedlings were transplanted at five sites representative of the restoration gradient (Figure 1). Plant performance and environmental characteristics were measured regularly during the first growing season. The hypotheses under which this experiment was conducted are: (1) abiotic conditions reflect restoration history, with mature restorations approaching conditions of uninvaded woodland, and (2) legumes perform better in mature restored woodlands and in sites with lower nitrogen and higher light availability.

Materials and methods

Site description and current restoration work

Mary Mix McDonald Woods is a 40-hectare, actively managed oak woodland located at Chicago Botanic Garden in Glencoe, Illinois. Prior to the establishment of the Garden, this area was an unmanaged park reserve heavily invaded by buckthorn and depauperate in native plant species (Steffen, personal communication). Fire suppression and herbivory by white-tailed deer and livestock led to loss of fire-adapted and grazing-susceptible native species (Rawinski and Square 2008). Similar to other woodland restoration sites in the Chicago region, the restoration goals of McDonald Woods include removal of invasive

buckthorn, thinning of over-abundant trees, reintroduction of fire regimes, and seeding to increase native herbaceous diversity. Invaded woodland restoration involves cutting buckthorn stands, applying of herbicide to prevent resprouting from cut stems, seeding, developing fuel load, and prescribed burning (Packard 2005, Solecki 2005). While restored sites contain high native species diversity and more light availability, they must be maintained by manual burning regimes and active control of weedy species (Packard and Ross 2005). Ongoing management since 1996 has resulted in multiple restoration sites at differing stages of maturity (Figure 1).

Legume seedlings and planting sites

Lespedeza and *Desmodium* were grown form field-collected seeds. *Desmodium* seed was stratified in damp sand at 4 °C for 4 weeks. *Lespedeza* seed was scarified in concentrated sulfuric acid (>95% H_2SO_4) for 8 minutes, rinsed thoroughly to remove residual acid, and stratified in damp sand at 4 °C for 2 weeks. Seeds were inoculated with species-specific *Rhizobium* inoculant (Prairie Moon Nursery, Winona, MN USA) at time of cold stratification, according to manufacturer instructions. Prepared seeds were sown in potting soil for germination in 48-cell trays. Seedlings were grown in a greenhouse at ambient humidity and light conditions for 8 weeks before transplanting. At time of planting, seedlings were checked for root nodules, though none were observed.

Desmodium and *Lespedeza* seedlings were transplanted in late May 2011 in pairs of one individual of each species, following established transplanting techniques for restoration

(Reinartz 2005, Steffen 2005). All planting sites were located along the ridge of a moraine spanning the length of the woodland, in well-drained areas identified as suitable for legume growth (Steffen, personal communication). Topographic variation on top of the moraine was minimal and unlikely to affect seedling between sites. Directional orientation of *Lespedeza* and *Desmodium* seedling pairs were haphazardly assigned, and seedlings were planted 1 m apart to preclude competition between seedlings (Casper and Jackson 1997, Smith 1975). Ten seedling pairs were planted in each of five restoration sites (Figure 1). Four previously invaded areas had been managed for invasive species and native biodiversity for 15, 9, 6, and 1 years prior to the time of the study. The 9-year site was originally comprised of two different sites (restored in 2001 and 2003), but has been managed as a single site since 2003, and was treated as a single restoration site for this study. Unmanaged "buckthorn" planting sites were located in a section of dense buckthorn that had not undergone restoration.

Because herbivory by deer and rabbits is a known problem for legumes, liquid herbivore repellant (Liquid Fence Deer & Rabbit Repellant, The Liquid Fence Company, Blakeslee, PA, USA) was used to prevent seedling loss (Muir and Bow 2008). Repellant was sprayed directly to leaves and stems until all leaves were visibly wet, and to the ground immediately surrounding the seedlings, according to manufacturer directions. Repellant application was repeated weekly for four weeks.

Soil nutrient analysis

Soil samples were collected from each planting pair location, with each sample collected as a pool of three subsamples: two from soil adjacent to each transplanted seedling, and one from soil between the two plants. Samples were collected using a hand trowel to a depth 10 cm. All samples were cleaned of roots and woody debris and homogenized. Moisture content of soil samples was measured gravimetrically as mass fraction lost after drying soil samples at 105 °C for 24 hours. Soil pH was measured electronically in a 50% w/w mixture of soil in water (EPA 2004). Soluble nutrients were extracted from soil in 2 M potassium chloride (KCl) under constant agitation for 2 hours. Extract supernatants were filtered and stored at 4 °C until analysis (Keeney and Nelson 1987). Soluble nutrient content of KCl extracts was measured with a SEAL AQ2 Automated Discrete Analyzer (SEAL Analytical, Mequon, WI, USA). Ammonium (NH_4), nitrate plus nitrite (NO_x), and ortho-phosphate (PO_4) were measured as mass fraction of analyte in dry soil (μg/g) using protocols adapted from standard methods (EPA 1993a, b, c).

Light availability

Light availability and canopy openness were measured by digital analysis of 180° hemispheric photographs captured using a circular fisheye lens (Becker et al. 1989, Frazer et al. 1999). All photographs were taken on the same day, with the camera positioned at plant height, leveled, and aligned to magnetic north. Images were pre-processed to mask anomalous bright spots from camera operators and reflections, and green and blue color channel data were discarded to enhance contrast between sky and leaf pixels. An optimal

black/white contrast threshold was selected for five representative images, and the median threshold value was applied to all images to ensure batch consistency. Image processing and light availability calculations were performed using Gap Light Analyzer version 2.0 (Frazer et al. 1999), yielding four measurements of light availability: percent sky openness, leaf area index (LAI), percent light transmission through the canopy, and light availability (Frazer et al. 1999, Stenberg et al. 1994). Percent sky openness was calculated directly from the pixel map, and the other measurements were derived from percent sky values and estimated solar transits calculated from the woodland geospatial coordinate and growing season dates. All four GLA variables were highly correlated, so only light availability was used for subsequent analyses, since it was assumed to be the environmental factor that most directly impacts plant growth at ground level. Light availability was expressed as photosynthetic light flux ($mol \cdot m^{-2} \cdot day^{-1}$).

Plant growth measurements

To measure plant growth and productivity, plant size data were collected weekly for six weeks after transplanting, then biweekly through the end of the growing season. *Desmodium* demographic measurements included: stem length, (distance from ground to the base of the tallest petiole, cm), leaf number, and pseudo-whorl coverage area (longest horizontal leaf-tip-to-leaf-tip distance multiplied by widest perpendicular distance, cm^2). *Lespedeza* demographic measurements included: stem length from base of primary stem to tip of longest branch, cm) and leaf number. At the end of the growing season, number of flowers (fruits plus pedicel scars) and number of mature fruits were counted. Relative growth rates

were calculated for stem and leaf growth (Hoffmann and Poorter 2002). Relative stem growth was calculated as $\frac{\ln S_{max} - \ln S_0}{t_{max} - t_o}$, where S_{max} is maximum stem length, S_0 is stem length at time of planting, and $t_{max} - t_0$ is the number of days between those two measurements, expressed as new stem growth per unit extant stem length per day ($cm \cdot cm^{-1} \cdot d^{-1}$). Relative leaf growth was calculated as $\frac{\ln L_{max} - \ln L_0}{t_{max} - t_o}$, expressed as new leaves per extant leaf per day ($leaf \cdot leaf^{-1} \cdot d^{-1}$). Plants were not exhumed at the end of the growing season, so nodulation data were not collected. Six legume pairs were excluded from this data set: three legume pairs in the 6-year restoration site that were encroached upon by an unidentified *Lonicera* species during most of the growing season, and three pairs in the buckthorn site that received atypically high light because of a treefall gap.

Statistical analyses

All data transformations and statistical analyses were performed using R version 2.15.0 (R Development Core Team 2012). A significance level of $\alpha = 0.05$ was used for all tests of significance. Linear modeling and multiple regression analyses were used to test for relationships between environmental variables, restoration, and plant performance metrics (Crawley 2005). Plant survival analysis was performed using the "survival" package implemented in R (Therneau 2012). Survival analysis is a statistical method that calculates survival models from sampled populations of individuals that may have died at varying times. These models predict mean survival time for a population and ANOVA tests can be applied to these models similarly to simple linear models. For this analysis, survival models were creat-

ed using interval-censored data, and parametric survival models assumed exponential distributions. Classification and regression tree (CART) analysis was used to identify interacting variables that influenced plant performance but did not have high explanatory power (Breiman et al. 1984). CART analysis is a statistical method that arranges explanatory variables in a hierarchy of decreasing influence on a single response variable. Complex trees are penalized when terminal branches (explanatory variables) do not improve the overall predictive power of the tree. These unnecessary variables are then "pruned" until the resulting tree is as small as possible without losing explanatory power. CART analysis was performed using the "rpart" and "rpart.plot" packages implemented in R (Milborrow 2011, Therneau and Atkinson 2012).

Results

Restoration age was not predictive of soil moisture, NH_4, PO_4, or pH, but NO_x ($R^2 = 0.110$, $p = 0.028$) and light ($R^2 = 0.153$, $p = 0.009$) were inversely proportional to restoration age (Table 1, Figure 2). Light availability was significantly higher in the 1-year site (16.05 $mol \cdot m^{-2} \cdot day^{-1} \pm 1.55$ se) than in other restoration sites (6.74 $mol \cdot m^{-2} \cdot day^{-1} \pm 0.36$ se), reflecting the lack of mature oak canopy at this site. There was no significant relationship between light availability and other restoration ages after removing the 1-year restoration area data ($R^2 = 0.023$, $p = 0.388$). NH_4 ($R^2 = 0.663$, $p < 0.001$), NO_x ($R^2 = 0.404$, $p < 0.001$), and PO_4 ($R^2 = 0.173$, $p = 0.005$) were all positively correlated with soil moisture (Figure 3). Soil pH also increased with soil moisture, but was most strongly associated with increased

NO_x (R^2 = 0.414, $p < 0.001$). Light availability was not strongly associated with moisture or soil nutrients, but was weakly correlated with pH (R^2 = 0.097, p = 0.040).

Relative leaf and stem growth for *Desmodium* and *Lespedeza* were not correlated with restoration age or environmental variables (Table 2). Lower soil moisture and NO_x were correlated with higher median *Desmodium* survival time (χ^2 = 6.32 on 2 df, p= 0.042, Table 3). Only two *Desmodium* plants bore fruit, precluding analysis of *Desmodium* fruit production. Increased *Lespedeza* fruit set was correlated with higher pH (R^2 = 0.118, p = 0.023), and with more light (R^2 = 0.113, p = 0.026, Table 2). *Lespedeza* median survival time was not correlated with restoration age or environmental variables.

All measures of plant performance were influenced by at least two environmental factors (Figure 4). Two environmental factors, soil pH and NO_x, had apparently contradictory effects of plant performance. Higher pH was associated with increased *Lespedeza* fruit set and *Desmodium* stem growth, but with reduced *Lespedeza* leaf growth for plants growing with higher PO_4 and lower NO_x. Increased NO_x was associated with increased *Desmodium* and *Lespedeza* leaf growth, but decreased *Desmodium* stem growth.

Discussion

Effects of woodland habitat restoration on environmental conditions

The hypothesis that abiotic conditions would reflect restoration history is not supported by the soil moisture, NH_4, PO_4, pH, or light data (*contra* Heneghan et al. 2006a, Klionsky

et al. 2011, Knight et al. 2007), but there is supporting evidence that NO_x is lower in more mature restoration sites. However, NO_x in the buckthorn-dominated site was unexpectedly low, and was closer to the 15-year site than the youngest restoration (*contra* Heneghan et al. 2006a). With the exception of NO_x, abiotic factors in this woodland did not seem to follow a predictable restoration trajectory. Soil moisture and nutrient concentrations were highly correlated, possibly in response to factors independent of restoration and not measured here. Increased light availability in the 1-year restoration site reflected oak canopy immaturity in this part of McDonald Woods, and light conditions were equivalent between the buckthorn-dominated site and restored sites of varying age elsewhere in the woodland.

These results are inconsistent with both field observations and past studies in similar woodlands. Because this study was conducted at a single site with few replicates and because environmental variables were measured at one time point, data are limited in scope and should be interpreted conservatively. Given that this study was designed to detect temporal changes over an ecologically short time scale, restoration-associated differences may have existed below the threshold of detection. In addition, these results were likely impacted by drought, which would have reduced apparent soil nutrient content through decreased nutrient motility and enzymatic activity (Graetz and Tongway 1986, Sardans and Peñuelas 2005). Elevated nitrogen from atmospheric deposition may have also masked variations in nitrogen availability between restoration sites (Fenn et al. 2003b, Gilliam 2006, NADP 2012). Additionally, nitrogen and phosphorus soil concentrations have been

shown to vary seasonally in other environments, with the lowest observed concentrations during the growing season (Cameron 1996, Taylor et al. 1982). It therefore possible that soil nutrient differences between restoration sites did exist but were not observed in this study.

Because *Desmodium* and *Lespedeza* are understory herbaceous species, all canopy images were captured at plant height. A limitation of GLA analysis is that these light measurements cannot differentiate between tree canopy, shrub canopy, and tall understory growth. GLA is therefore blind to differences in canopy complexity or light quality. However, it is also blind to differences in time of day and weather conditions that can strongly affect direct light measurements. Though these sites yielded the same absolute amount of light at ground level at the time of measurement, they may have provided differences in light quality through the growing season (Smith 1982). Buckthorn flushes earlier and remains foliated later than the oak canopy and co-occurring shrubs (Knight et al. 2007). Because the canopy images were all recorded in mid-August when the oak canopy was fully developed, phenological differences between restored and unrestored sites were not captured. This study investigated abiotic factors and plant community data were not addressed, though competition with different plant communities may have strongly affected legume performance. Previous work has shown that there was more bare ground under buckthorn, whereas mature restored areas more understory cover and more diverse grass and forb communities (Larkin, et al., *in review*).

Woodland legume response to restoration and abiotic factors

The data do not support the hypothesis that legumes perform better in older restoration areas. However, *Desmodium* survived longer in drier soil and with lower nitrogen, both of which are target conditions for restoration of buckthorn-invaded areas (Heneghan et al. 2006a, Kline 2005b). *Lespedeza* yielded more fruits in soils with higher pH and more light, but there were no significant predictors for increased *Lespedeza* survival. Neither restoration age nor any measured environmental factor was correlated with stem or leaf growth of either legume species. CART data indicated complex interactions between environmental variables and legume performance, but did not identify environmental conditions for optimal legume growth and reproduction. Because legume performance was affected by multiple factors, sometimes with contradictory effects, this study did not identify a set of field conditions ideal for optimal legume performance.

These plants were not removed from the field at the end of the season, so nodulation data are not available. Because these seedlings were inoculated with commercially sourced inoculants and were planted in sites where no other legumes were growing, it is possible that they were unable to fix atmospheric nitrogen (see Chapter 2, Results). However, it is also possible that compatible nodule-forming bacteria were already available in the soil at the planting sites and that these plants were nodulated.

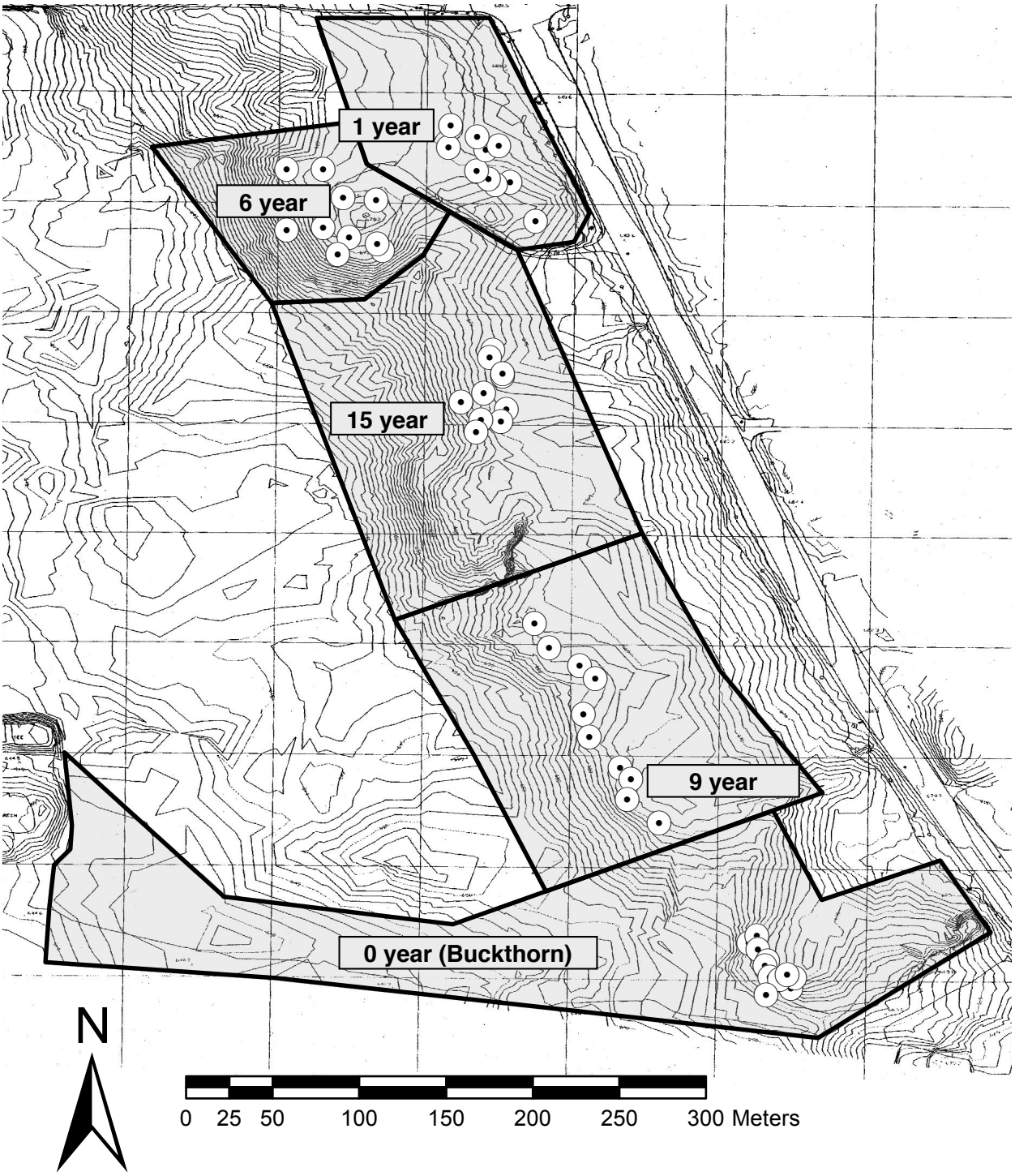

FIGURE 1: Map of Mary Mix McDonald Woods and restoration areas. Numeric labels indicate the number of years each restoration site had been under invasive species management at the time of planting. Point markers indicate planting locations with one *Desmodium* and one *Lespedeza* at each point.

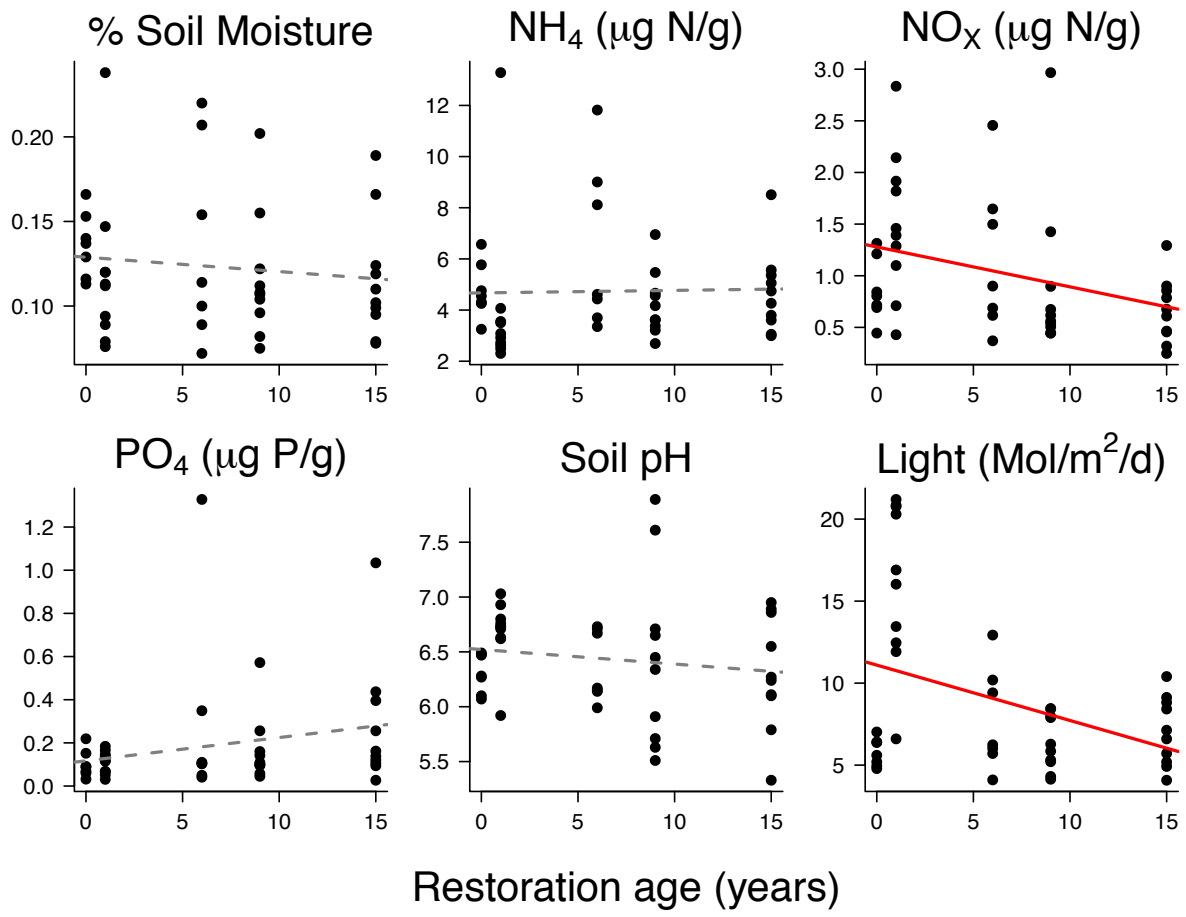

FIGURE 2: Six environmental variables plotted as functions of restoration age with simple linear model trend lines. Zero-year-old sites represent unrestored buckthorn. Solid trend lines in red indicate statistically significant relationships ($p \leq 0.05$), while dashed trend lines in grey indicate insignificant relationships ($p > 0.05$, see Table 1).

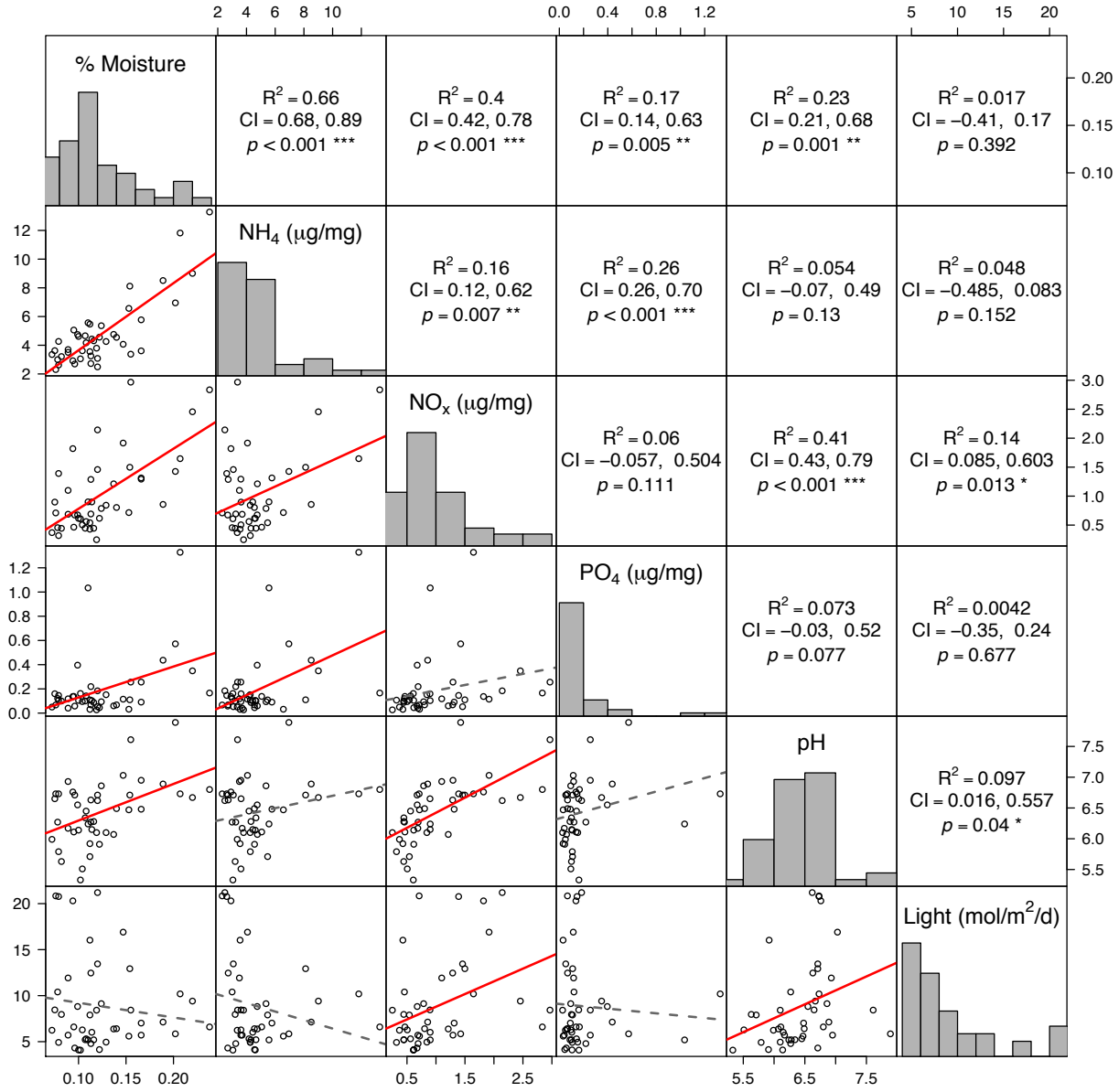

FIGURE 3: Pairwise correlation of environmental variables, with correlation statistics for simple linear regressions and predicted trend lines. Solid trend lines in red indicate statistically significant relationships ($p \leq 0.05$), while dashed trend lines in grey indicate insignificant relationships ($p > 0.05$). Significance indicators: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

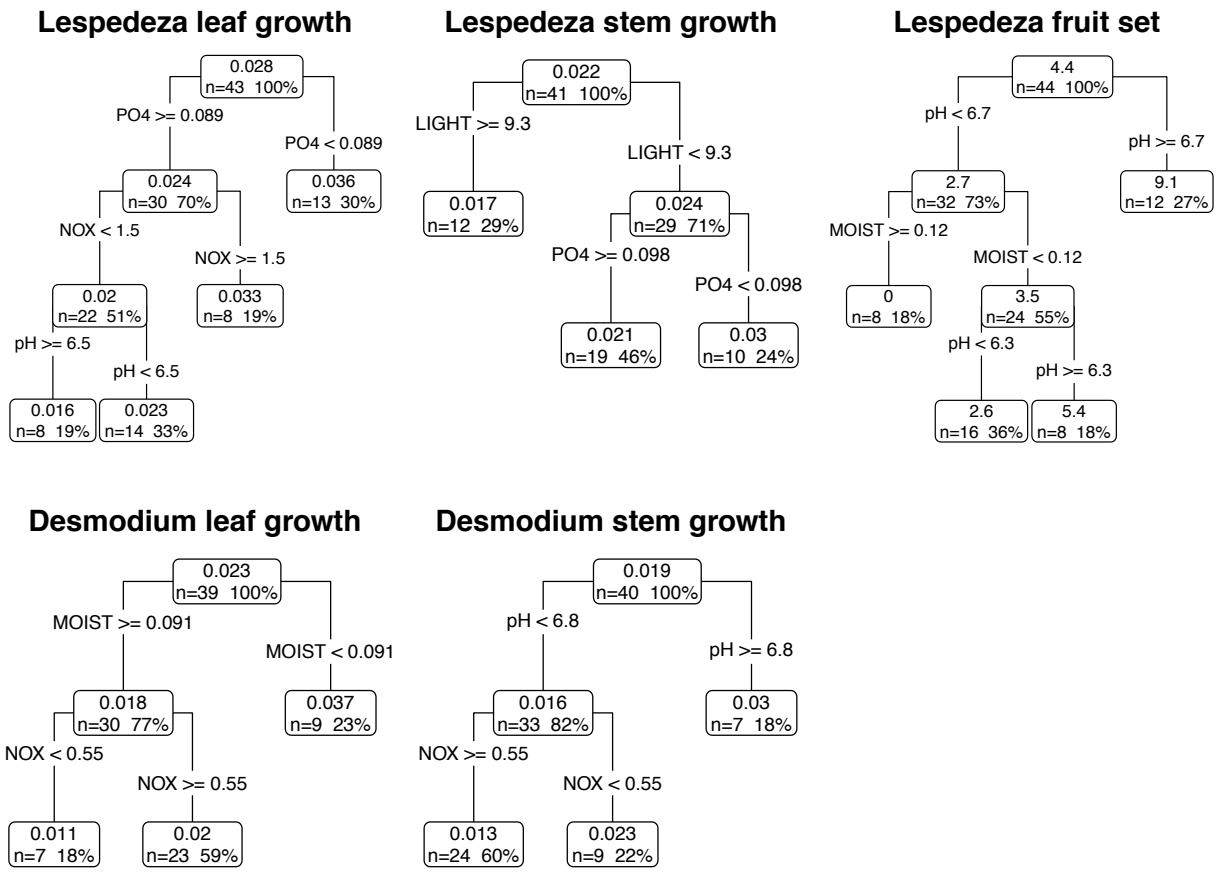

FIGURE 4: Pruned regression trees for plant performance metrics. For each tree, higher performance values are on the right. Nodes indicate group mean value, number of observations in that node, and percent of total observations represented by that node.

TABLE 1: Simple linear regression model results for six environmental variables as a function of restoration age.

Environmental Variable	R^2	F statistic (DF=1,42)	*p* value
% Moisture	0.014	0.604	0.441
NH_4	0.001	0.024	0.878
NO_x	0.110	5.201	0.028 *
PO_4	0.061	2.708	0.107
pH	0.022	0.960	0.333
Light	0.153	7.598	0.009 **

Significance indicators: * $p < 0.05$, ** $p < 0.01$

TABLE 2: Simple linear model results for plant productivity measures as functions of restoration age and environmental variables.

Plant performance metric	Predictive variable	R^2	F statistic	df	*p* value
Desmodium					
Relative leaf growth	Restoration age	< 0.001	< 0.001	1, 37	0.989
	Soil moisture	0.064	2.535	1, 37	0.120
	Ammonium	< 0.001	0.010	1, 37	0.923
	Nitrate/nitrite	0.030	1.159	1, 37	0.289
	Orthophosphate	0.014	0.528	1, 37	0.472
	Soil pH	0.056	2.196	1, 37	0.147
	Light availability	0.034	1.300	1, 37	0.262
Relative stem growth	Restoration age	0.057	2.292	1, 38	0.138
	Soil moisture	< 0.001	0.010	1, 38	0.921
	Ammonium	0.022	0.853	1, 38	0.362
	Nitrate/nitrite	0.003	0.105	1, 38	0.747
	Orthophosphate	0.004	0.152	1, 38	0.699
	Soil pH	0.007	0.282	1, 38	0.598
	Light availability	0.000	0.003	1, 38	0.958
Lespedeza					
Relative leaf growth	Restoration age	0.069	3.037	1, 41	0.089
	Soil moisture	0.002	0.076	1, 41	0.784
	Ammonium	0.006	0.229	1, 41	0.635
	Nitrate/nitrite	0.001	0.034	1, 41	0.855
	Orthophosphate	0.083	3.704	1, 41	0.061
	Soil pH	0.030	1.271	1, 41	0.266
	Light availability	0.003	0.134	1, 41	0.716
Relative stem growth	Restoration age	0.009	0.372	1, 39	0.546
	Soil moisture	0.028	1.118	1, 39	0.297
	Ammonium	0.003	0.122	1, 39	0.729
	Nitrate/nitrite	0.018	0.707	1, 39	0.406
	Orthophosphate	0.007	0.265	1, 39	0.610
	Soil pH	< 0.001	0.008	1, 39	0.929
	Light availability	0.022	0.893	1, 39	0.350
Fruit set	Restoration age	0.031	1.340	1, 42	0.254
	Soil moisture	0.024	1.048	1, 42	0.312
	Ammonium	0.072	3.276	1, 42	0.077
	Nitrate/nitrite	0.009	0.383	1, 42	0.539
	Orthophosphate	0.001	0.039	1, 42	0.844
	Soil pH	0.118	5.609	1, 42	0.023 *
	Light availability	0.113	5.334	1, 42	0.026 *

Significance indicators: * $p < 0.05$

TABLE 3: Parametric survival model results for legume survival as a function of restoration age and environmental variables.

Plant performance metric	Environmental variable	χ^2	df	*p* value
Desmodium survival	Restoration age	0.392	1	0.531
	Soil moisture	4.491	1	0.034 *
	Ammonium	2.302	1	0.129
	Nitrate/nitrite	5.734	1	0.017 *
	Orthophosphate	1.752	1	0.186
	Soil pH	3.509	1	0.061
	Light availability	0.534	1	0.465
Lespedeza survival	Restoration age	0.029	1	0.864
	Soil moisture	0.905	1	0.341
	Ammonium	0.150	1	0.699
	Nitrate/nitrite	0.369	1	0.543
	Orthophosphate	0.483	1	0.487
	Soil pH	< 0.001	1	0.995
	Light availability	1.580	1	0.209

Significance indicators: * $p < 0.05$

Chapter Two: Greenhouse Experiments

Introduction

Desmodium and *Lespedeza* are both facultative nitrogen fixers, and other species of *Desmodium* and *Lespedeza* have been reported to exhibit promiscuity with nodule-forming bacterial strains (Gu et al. 2007, Jha et al. 1995, Parker 1999, Yao et al. 2002). However, studies of microsymbiont diversity show that these legumes typically host *Bradyrhizobium* species. Commercially available seed sources are often shipped with general-purpose non-specific *Rhizobium* inoculant intended for use with most legume genera including both *Desmodium* and *Lespedeza*. While commercial sources claim that these inoculants yield healthy nodules, seed suppliers in the past have provided species-specific inoculants that are no longer available. There are concerns about the compatibility and efficacy of nonspecific cultures (Steffen, personal communication). It is possible the legume symbiont promiscuity in *Desmodium* and *Lespedeza* is restricted to the *Bradyrhizobium* genus and that *Rhizobium*-based inoculants are not able to form nodules.

Even if nonspecific inoculants are performing as expected, increased soil nitrogen availability can selectively favor non-nitrogen-fixing forbs and grasses in woodland restoration sites (Fenn et al. 2003a, Gilliam 2006). Increased soil nitrogen can reduce root nodulation and stimulate growth of neighboring plants in greenhouse experiments, negating the advantages conferred by nitrogen fixation under low nitrogen conditions (Skogen et al. 2011). Previous studies have shown that increased nitrogen directly impairs root nodule formation in agricultural legumes (Eardly et al. 1985, Imsande 1986) and native woodland

legumes (Skogen et al. 2011), as well as in non-legume actinorhizal nitrogen fixers (Kohls and Baker 1989). Given that oak woodlands have historically been nitrogen-limited (Reed et al. 2007, Vitousek and Howarth 1991), and that restoration efforts frequently make use of fast-growing, nutrient-hungry native grasses to suppress invasive species (Packard 2005, Sauer 1998, Solecki 2005), the compounded effects of competition and nitrogen deposition pose potentially serious risks to nascent legume populations in woodland restorations.

To investigate inoculant efficacy, *Desmodium* and *Lespedeza* seedlings were grown in a growth chamber with each of four inoculant treatments: no inoculant, species-specific inoculant, nonspecific inoculant, or field soil collected from the rhizospheres of healthy, nodulated, conspecific legumes. Species-specific inoculants were hypothesized to yield more active nodules than nonspecific inoculants. To determine the combined effects of nitrogen addition and competition on legume performance, a factorial experiment was conducted in a greenhouse using three nitrogen deposition rates and three competition treatments. Increased nitrogen availability was hypothesized to counteract the competitive advantage of nitrogen fixation, resulting in reduced legume performance.

Materials and methods

Inoculant trials

Commercially sourced species-specific inoculants (Prairie Moon Nursery, Winona, MN USA) and nonspecific legume inoculant (Taylor Creek Restoration Nursery, Brodhead,

WI) were tested for nodulation efficacy in greenhouse-grown seedlings. *Lespedeza* seeds were scarified as previously described (see Chapter 1 methods). Seeds were sterilized in 10% v/v household bleach in water with a few drops of dishwashing detergent for 10 minutes (Sauer and Burrows 1986). *Desmodium* and *Lespedeza* seeds were germinated at room temperature in wetted filter paper without cold stratification. Within one day of germinating, sprouted seeds were coated with a slurry of powdered inoculant in sterile water and planted 1–2 mm below soil level (Baskin and Baskin 2001). Both species were planted into 2 48-cell trays (96 replicates total) for each of 3 inoculant treatments: sterile water only, specific inoculant, and nonspecific inoculant. This experiment was replicated twice, using compost-based potting soil or sterile sand. Seedlings were grown in an incubation chamber with conditions simulating average May photoperiod, temperature, and humidity cycles for Glencoe, IL: 14.5 hour photoperiod, 24.2 °C/8.2 °C daytime/nighttime temperature cycle, 43.8 %/85.2% daytime/nighttime humidity cycle (NOAA 2011). After six weeks (potting soil) or three weeks (sand) of growth, plants were harvested (above- and belowground biomass) and root nodules were counted (Johnston and Beringer 1975). As a control to ensure that available seeds would nodulate under field conditions, scarified and sterilized seeds were planted in 3.8 L pots filled with sand topped with field soil collected from the rhizospheres of established populations of *Desmodium* and *Lespedeza*. Control seedlings were harvested after nine weeks of growth.

Nitrogen deposition and competition

The effects of competition and nitrogen deposition on plant productivity and nodule formation were tested in a full-factorial greenhouse experiment that included three competition treatments, three nitrogen deposition treatments, and both legume species. There were 12 replicates for each nitrogen and competition treatment pairing except for grass competition treatments which had 8 replicates due to poor grass germination. Seedlings grown in 3.8 L pots containing 9:1 sand:vermiculite (by volume) with a small amount (less than 1%) of field soil to provide trace minerals and nutrients. Pots were placed under 40% shade cloth in a greenhouse with ambient humidity and natural light only, and were watered as needed. Pots were grouped in blocks by nitrogen treatment, but within treatment blocks, competition treatments and legume species were haphazardly mixed and rotated twice during the experiment to avoid greenhouse position effects.

Elymus villosus Muhl. (Silky Wildrye, henceforth *Elymus*) is a grass frequently used in early woodland restoration to rapidly establish ground cover (Sauer 1998). It was selected as the competitor species for these experiments because it is readily available, easy to propagate, and commonly used in restoration work at McDonald Woods, making it a likely competitor for future legume populations. *Lespedeza* and *Desmodium* seeds were sterilized and scarified as previously described. *Elymus* seeds did not require scarification or stratification. Legume seeds were inoculated with species-specific, commercial inoculant. Seeds were germinated, sown in 128-cell plug trays, and grown in an incubation chamber for three weeks to establish roots before transplanting. There were three competition treat-

ments: noncompeting plants (one legume only), conspecific competition (two legumes together), and interspecific competition (one legume with one grass), for both legume species and all nitrogen deposition treatments. Paired plants were spaced approximately 3 cm apart in the center of the pot to encourage belowground interaction within the growing medium.

Nitrogen was applied as urea with added urease inhibitors to prevent enzymatic denitrification and leaching (46-0-0 UFLEXX, Agrotain International LLC, St. Louis, Missouri, USA) dissolved in water. The urease inhibitor enzymes in UFLEXX slow urea hydrolysis and prevent nitrification of ammonium, ensuring that most of the nitrogen delivered remains bioavailable in the soil (Soldat et al. 1999). Three nitrogen deposition treatments were used: "control" replicates received weekly applications of water only, "low" replicates were supplemented with nitrogen equivalent to current US deposition rate: 6.4 $kg \cdot ha^{-1} \cdot y^{-1}$ (NADP 2012), and "high" replicates received application levels recommended by the manufacturer for use on turf grass: 284 $kg \cdot ha^{-1} \cdot y^{-1}$. Urea application rates were derived from these deposition rates for eight weekly applications onto a potting soil area of 31.92 cm^2 (8-inch circular pot): high deposition, 116.60 mg/pot/week; low deposition, 2.65 mg/pot/week. Nitrogen treatments were applied as 6 mL of fertilizer solution: 43.19 g urea/L for high deposition, 0.983 g urea/L for low deposition, sprayed directly to the soil surface in each pot. Immediately after application, all pots were lightly watered to ensure penetration of nitrogen into the growing medium, care was taken to ensure that no water escaped from the bottom of the pots due to excess watering. To confirm the efficacy of ni-

trogen addition, soil samples were collected from randomly selected pots 24 hours after nitrogen addition and soluble nitrogen content was measured by the same KCl extraction method reported in Chapter 1. After 13 weeks of growth in the greenhouse, plants were harvested (above- and belowground biomass), and where possible, roots from competing plants were separated from each other. Inseparable root systems were processed together as a single, combined root mass. Roots were cleaned of remaining potting soil, and root nodules were counted and visually inspected for viability. Nodule viability was confirmed by observation of pink coloration in crushed sample nodules (Hansen 1994). Above- and belowground biomasses were dried for 48 hours before weighing.

Statistical analyses

All data transformations and statistical analyses were performed in R version 2.15.0 (R Development Core Team 2012). Only plants that survived the entire experimental period were included in plant performance analyses, and combined root masses that could not be separated were excluded from belowground data. Multi-factor Analysis of Variance (ANOVA) was used to test for effects of competition and nitrogen deposition on above- and belowground biomass, and above/below mass ratio. Since sample sizes varied due to plant loss and exclusion of data, Tukey's Range Tests were used to determine differences between group means.

Results

No root nodules were observed on *Desmodium* and *Lespedeza* seedlings when grown with either nonspecific or species-specific inoculant. However, nodules were observed in both species after growth in field soil harvested from wild legume populations (Figure 5). Similarly, there were no nodules observed in plants from nitrogen deposition and competition experiments, all of which has been inoculated with species-specific inoculant.

Within one day of UFLEXX application, soil NH_3 concentration was elevated in pots receiving high nitrogen deposition levels, but not in low nitrogen pots ($F_{2,18} = 13.57$, $p < 0.001$). NO_x was elevated in both high and low nitrogen plots ($F_{2,18} = 3.84$, $p = 0.041$). Nitrogen levels were not measured more than one day after application, but UFLEXX is reported to provide slow-release nitrogen for at least 14 days after application (Agrotain 2012). Across all competition treatments, plants of both species receiving high levels of nitrogen were less likely to survive through the end of the experimental period (*Desmodium*: $F_{2,129} = 35.55$, $p < 0.001$; *Lespedeza*: $F_{2,129} = 44.54$, $p < 0.001$).

Competition was not a significant factor in *Desmodium* growth, but nitrogen addition affected aboveground ($F_{2,78} = 7.143$, $p < 0.001$) and belowground ($F_{2,69} = 11.724$, $p < 0.001$) biomass as well as above/below allocation ($F_{2,69} = 6.477$, $p = 0.003$, Table 4, Figure 6). High nitrogen replicates were smaller (above- and belowground biomass) than low nitrogen or control replicates. Above/below mass ratio for high-nitrogen treatments was higher than low nitrogen but not higher than controls. It should be noted that above/below ratio data

were relatively variable for high-nitrogen treatments, and that these data represent fewer plants than the lower nitrogen addition treatments due to *Desmodium* mortality at high nitrogen levels.

Aboveground *Lespedeza* biomass was affected by both competition ($F_{2,98}$ = 3.205, p = 0.045) and nitrogen addition ($F_{2,98}$ = 6.137, p = 0.003), while belowground biomass ($F_{2,74}$ = 9.148, p < 0.001) and above/below ratio ($F_{2,74}$ = 4.715, p = 0.012) were affected by nitrogen addition only (Table 4, Figure 6). Aboveground biomass of non-competing plants was higher than interspecific competitors, but not higher than intraspecific competitors, while interspecific and intraspecific groups were equivalent. Above- and belowground biomass of high nitrogen replicates was lower than low nitrogen or control treatments. Above/below mass ratio in high nitrogen treatments was higher than in low nitrogen but not lower than controls, while low nitrogen and controls were equivalent.

Discussion

Commercially sourced inoculants did not yield root nodules

As no root nodules were observed with either commercial inoculant, the hypothesis that species-specific products would perform better than nonspecific inoculant was not supported. However, since nodules did grow with field soil, it can be concluded that neither specific nor nonspecific inoculant was effective with *Desmodium* and *Lespedeza*. It is likely that the strains distributed in commercial inoculants are not able to nodulate with these species. That inoculant strains are labeled as *Rhizobium*, whereas multiple diversity stud-

ies identify *Desmodium* and *Lespedeza* symbionts as *Bradyrhizobium*, lends support to this hypothesis (Gu et al. 2007, Yao et al. 2002). Culturing techniques ensure that isolates are harvested from within active nodules, but it is possible for multiple bacterial species to be collected in this way (Johnston and Beringer 1975). Because only a small fraction of soil microbes can be cultured in vitro, the isolates from which commercial inoculants were derived may have been the wrong bacterial genus (Janssen 2006). Field soil replicates were not repeated using other soils from areas without legumes. Therefore, this experiment was simply a positive control to confirm nodule formation ability, and not a test of the general nodulation capacity of native woodland soil microbes. For future restoration efforts, it would be important to determine whether the extant soil microbes in target legume reintroduction sites can form root nodules. If so, there would be no need for additional inoculant, thereby avoiding the need to excavate soil from established legume populations.

Legume response to competition and nitrogen deposition

There was no main effect of competition on *Desmodium* performance, but for *Desmodium* plants at low nitrogen deposition rates, above- and belowground biomass were greater in plants grown with grass than in noncompeting plants. This difference was not observed at high nitrogen rates or control replicates, and may be spurious, given limited sample number. Because mean *Desmodium* biomass was the same in low nitrogen and control pots, and no nodules were observed in any of these seedlings, increased biomass was likely not a result of higher nitrogen availability. For all nitrogen treatments, *Lespedeza* aboveground biomass was higher in plants grown alone than those grown with grass. While not observed

for belowground biomass, this effect supports the hypothesis of competition reducing performance with this species.

High levels of nitrogen lowered biomass at all competition levels for both legumes and for grass competitors. While these data support the hypothesis that legumes perform better at lower nitrogen deposition rates, the lack of root nodules and unnaturally high deposition rate suggest a direct toxicity effect from urea or ammonia (Bremner 1995, Britto and Kronzucker 2002). These plants abscised leaves more frequently, and root tips seemed dried or withered when harvested. High-nitrogen plants also allocated more biomass aboveground. Although it is possible that increased aboveground allocation reflected preferential aboveground growth given abundant nutrient availability, this could also indicate loss of belowground biomass from tissue damage. Because none of the plants nodulated, supplied fertilizer was the only nitrogen source available beyond background levels of nitrate in the substrate. The assumption that legumes cope with competition for nitrogen by fixing atmospheric N_2 directly was therefore not applicable in this experiment. A larger scale experiment with more replicates across a broader range of nitrogen deposition would be necessary for a more conclusive analysis.

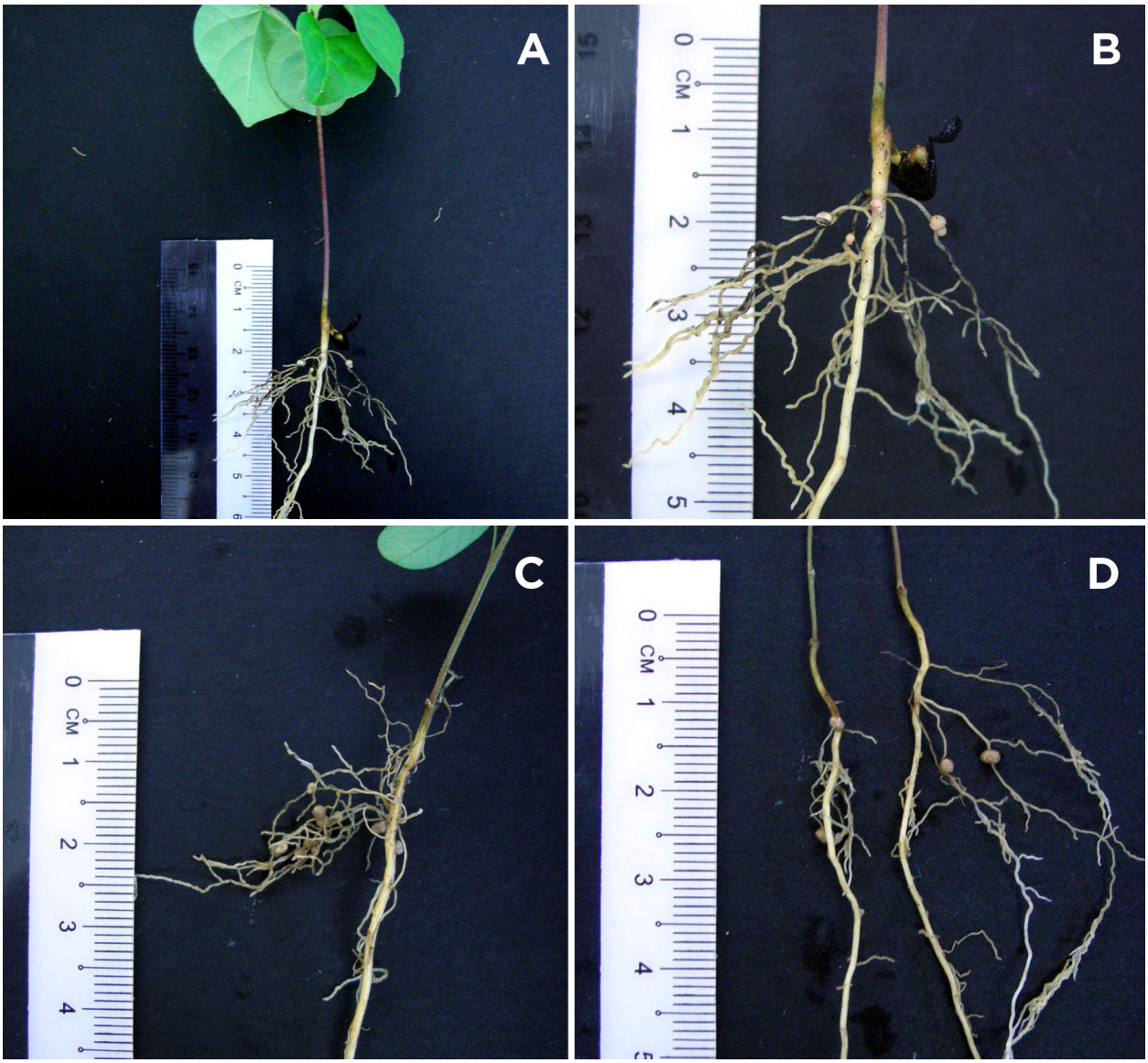

FIGURE 5: Root nodules growing on *Desmodium* (A and B) and *Lespedeza* (C and D) seedlings grown in field soil harvested from conspecific rhizospheres.

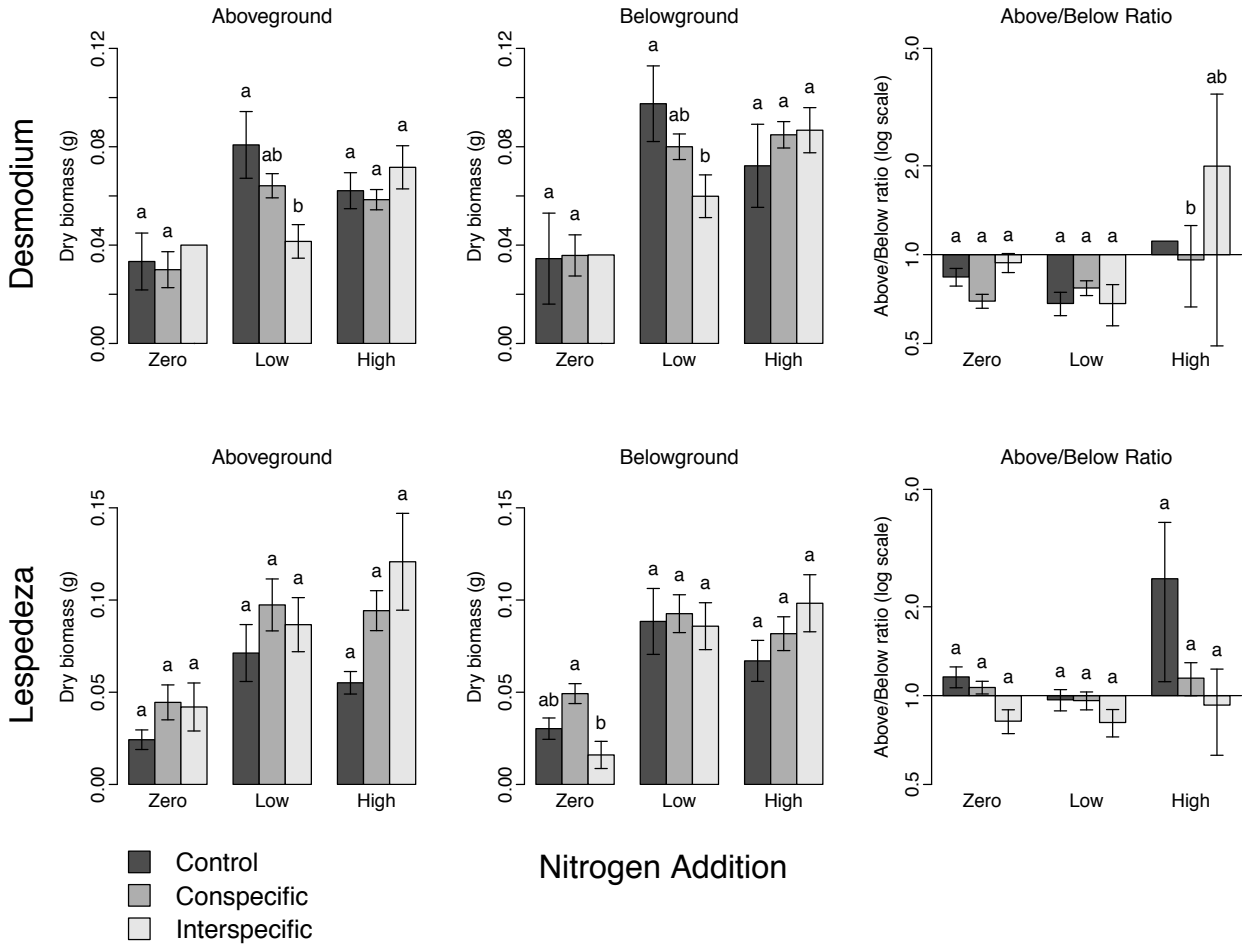

FIGURE 6: Effects of competition and nitrogen addition on legume biomass. Significance groups are based on Tukey's Range Tests. Group labels indicate differences between competition levels within each nitrogen addition treatment. Bars without significance letters or error bars represent single observations (sample variance not applicable).

TABLE 4: Multi-factor ANOVA results for greenhouse experiments.

Response variable	Factor	df	F statistic	*p* value
Desmodium				
Aboveground biomass	Competition	2, 78	0.702	0.499
	Nitrogen	2, 78	7.143	0.001 **
	Competition × Nitrogen	4, 78	2.711	0.036 *
Belowground biomass	Competition	2, 69	0.197	0.822
	Nitrogen	2, 69	11.724	<0.001 ***
	Competition × Nitrogen	4, 69	1.602	0.184
Above/below ratio	Competition	2, 69	2.023	0.140
	Nitrogen	2, 69	6.477	0.003 **
	Competition × Nitrogen	4, 69	2.988	0.025 *
Lespedeza				
Aboveground biomass	Competition	2, 98	3.205	0.045 *
	Nitrogen	2, 98	6.137	0.003 **
	Competition × Nitrogen	4, 98	0.632	0.641
Belowground biomass	Competition	2, 74	1.152	0.322
	Nitrogen	2, 74	9.148	<0.001 ***
	Competition × Nitrogen	4, 74	0.898	0.470
Above/below ratio	Competition	2, 74	2.902	0.061 ·
	Nitrogen	2, 74	4.715	0.012 *
	Competition × Nitrogen	4, 74	3.693	0.009 **

Significance indicators: · $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Recommendations for woodland legume restoration

Based on available evidence, the following recommendations are presented for future woodland legume restoration efforts in the region.

- Use deer exclosures. Given previously observed damage from deer browse on unprotected legumes, and in light of high survival rates among repellant-protected plants in field experiments, restorationists should use small, localized deer exclosures to protect new legume populations during the first growing season. This is a relatively low-cost, low-maintenance measure to protect incipient populations of young plants from loss to herbivory.

- Transplant soil in lieu of direct inoculation. The stark contrast in nodulation between commercially purchased legume inoculant and field-harvested soil emphasizes the importance of supplying the correct microsymbionts to legume seedlings. While harvesting soil from existing legume populations is time and labor intensive as well as a potentially serious habitat disturbance, it may be needed to ensure root nodulation in new legume populations, particularly where those populations are geographically isolated. Once a healthy patch is established at a particular restoration site, it should be relatively easy to transplant soil and healthy, young plants to nearby seeding sites to further propagate soil bacteria communities. Development of inoculant cultures is costly and may not be possible if *Rhizobium/Bradyrhizobium* species are difficult to grow *in vitro*. A simple experiment to test whether viable

nodule forming bacteria exist in the field may preclude the need for soil transplant altogether.

- Plant *Desmodium* in upland areas of older restorations. *Desmodium* had better survival rates with lower nitrogen and soil moisture. Therefore, new populations should be planted in upland sites with good soil drainage. Given that nitrate levels decreased with restoration age, *Desmodium* should be introduced in older restoration sites that have lower nitrogen availability.

- Plant *Lespedeza* in sunny, high-pH areas of older restorations. *Lespedeza* produced more fruit with increased light availability and in soils with higher pH. Therefore, it may be beneficial to seed and transplant in sunny patches under gaps in the canopy. Given that buckthorn invasions are associated with soil acidification, it may be best to plant or seed *Lespedeza* in more mature restorations in areas where pH is higher.

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