RESEARCH

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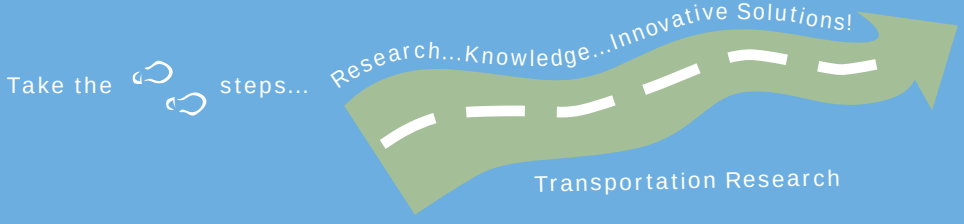
Improved Methodologies for the Inoculation of Prairie Legumes in Roadside/ Revegetation Settings



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Response to five different inoculation treatments has been determined in a three-year-old prairie area established at the Becker Sandplain Experiment Station in Fall 2004. Seed inoculation was generally ineffective, but overall legume numbers and biomass in the prairie restorations were enhanced by both soil-applied granular and cover-crop applied inoculants, with soils collected from the prairie areas in 2007 also showing marked improvement in the soil quality traits Microbial biomass C and N as a result of inoculation. When *Dalea* rhizobia were recovered from soil in the different prairie plots, and identified using BOXA1R-PCR, only 2% of the strains from the seed inoculation treatment identified with the inoculant strains, whereas 53% -100% of the rhizobia from soil in the other treatments identified with these strains. *Dalea* plants inoculated with rhizobia recovered from soil and identifying with the inoculant strains outyielded those inoculated with non-inoculant strains by more than 100%. In contrast, when slow-growing rhizobia from *Desmodium canadense* were recovered from soil and characterized, only 13.7% of the strains identified with the inoculant strains used. Most were not intended for *Desmodium per se* but identified with the inoculant strains intended for *Chamaecrista fasciculata,* the legume species most evident in the first season after planting. Inoculation with high potency granular soil-applied inoculants improves both the nodulation and establishment of prairie legumes, and the quality of the prairie, but species differences in response to inoculation require further study, particularly relative to host establishment pattern, host/strain compatibility, spatial variability in soil and environmental influences

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Improved Methodologies for the Inoculation of Prairie Legumes in Roadside/Revegetation Settings

Final Report

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EXECUTIVE SUMMARY

Prior to settlement, there were more than 18 million acres of prairie in western and southern Minnesota; today less than 0.5% of that remains, much of it in fragmented pockets of vegetation and in various stages of degradation. Loss or deterioration of prairie raises concerns of genetic and soil erosion, and of carbon loss and consequences for global warming. This has spurred significant efforts at reconstruction, with the Minnesota Department of Transportation (MNDOT) establishing native grassland areas alongside roadways, seeding areas around rest stops on the major highways with prairie plants, and undertaking extensive restoration with native plants for wetland mitigation. Contributing to the decision to re-establish prairie areas is that while they are visually attractive, only limited attention is needed post-establishment.

Nitrogen is deficient in many natural environments, and if prairie areas are to be sustainable, they must maintain soil nitrogen at levels adequate for prairie plant growth, and for the ground cover needed to minimize erosion and degradation of roadside and revegetation areas. Because of their ability to fix nitrogen in symbiosis, legumes have long been recognized for their importance in natural ecosystems, and MNDOT includes a number of indigenous legumes in roadside revegetation and wetland reconstruction plant mixes. Unfortunately, not all land areas contain rhizobia suitable for use with these legumes and inoculation with appropriate rhizobia is often necessary. The successful inoculation of prairies or prairie legumes presents problems not encountered in agricultural situations. Problems include lack of information on the *Rhizobium* requirements of the legumes used; exposure of host legumes and rhizobia to freezing and thawing following fall seeding; low seeding rate for the legumes used, and irregular germination of inoculated legumes in the following season, with limited opportunity for build up of rhizobia in soil.

The studies reported here had three thrusts;

- The use of cereals as a carrier of inoculant rhizobia for prairie legumes, and as a possible beneficiary from the effects of plant growth promoting compounds elaborated by these organisms.
- To evaluate different methods for the effective inoculation of prairie legumes in a restoration setting.
- To determine the feasibility for the inoculation of legumes growing in heavily fertilized seed blankets.

A field study initiated at Becker, MN in Fall 2004 compared 33 cereal cultivars for differences in the ability to support rhizobia in their rhizosphere, and for spread of these inoculant strains in soil over time. Cultivar differences were evident, with Roughrider, Wahoo, Wintergrazer, Wesley and Agassiz giving good growth and nodulation of subsequently seeded legumes. In a comparison of the wheat cultivars Roughrider and Oklee, counts of *Dalea* rhizobia from the Roughrider rhizosphere were substantially greater than for Oklee, with little evidence of inoculant strain interaction. In a third, glasshouse study rhizobial numbers for the *Dalea* strain UMR6808 showed little effect of mixed strain inoculants, whereas subsequent nodulation of *Desmodium* by rhizobia from a mixed culture was inhibited. A side product of these studies was a synthetic selective medium for the isolation of *Rhizobium* direct from soil. More studies are needed, but this medium could be extremely useful in comparing legume-dependent and direct isolations of rhizobia from soil, and in making plate counts from soil and rhizosphere.

The main thrust of this project was to improve methods of legume inoculation, and so the nodulation and nitrogen fixation of the prairie legumes used in restoration, and the growth and development of associated prairie plants that must also depend on this N for their function. Prairie areas established at the University of Minnesota Sandplain Experiment Station, Becker, MN in 2004 used a high diversity seed mix that included the legume species *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea candida* and *D. purpurea, Desmodium canadense* and *Lespedeza capitata.* Following establishment, the prairie areas were all mowed and raked, or burned annually to induce nitrogen deficiency, and favor legume establishment.The five treatments imposed were:

- Uninoculated
- Seed inoculated with a mix of nodule bacteria appropriate to the legumes seeded
- Inoculated with the same bacteria but in a granular clay-based formulation applied at 10 kg ha^{-1}
- As for the previous treatment, but using a granular peat-based inoculant
- Winter wheat seed inoculated with the mix of rhizobia as in treatment 2, but seeded as a cover crop prior to prairie establishment

There were four replications of each treatment.

From Spring 2005 to Spring 2008, each plot area was monitored for plant and species numbers and plant development; for evidence that inoculation method influenced inoculant strain recovery over time and strain persistence in soil; and for soil, plant and microbial traits likely to be affected by inoculation method.

Major findings pointing to the benefits of effective nodulation, and the need for higher than normal inoculation rates included:

- Uninoculated plots or plots that were seed inoculated with relatively low numbers of rhizobia applied, showed very limited change in soil microbial biomass N over the three years from prairie establishment. In contrast treatments employing granular inoculant formulations, or with inoculant applied via the wheat cover crop, showed increases in microbial biomass N of up to 37.5% compared to immediately adjacent agricultural fields. Soils inoculated with a granular clay based inoculant increased from 6.96 to 9.57 ppm microbial biomass N over the three years of this trial. This is still significantly less than found in the adjacent 1999-seeded prairie where microbial biomass N is now 18.27 ppm, evidence that improvement in this important soil indicator will be slow.
- Number of legumes per plot and biomass of legumes per quadrat in the third year after establishment varied from 68 and 1.21g in the seed inoculated treatment to 133 and 5.15 g in the granular clay-based treatment. Over the course of the study there was marked decrease in weed species and corresponding development in prairie forbs and grasses.
- 315 dalea rhizobia trapped from soil in 2007 and fingerprinted using BoxA1R-PCR could be divided into eight clusters, with four of these showing similarities to the inoculant strains used. While only 2% of strains recovered from the seed inoculated treatment showed homology to the inoculant rhizobia, 92-100% of strains in the granular peat and wheat-cover crop treatments showed homology to these strains. Further, when strains taken at random from those trapped from soil were tested for ability to enhance the growth of *Dalea purpurea* in magenta units, plants inoculated with strains subsequently identified as similar to the inoculant cultures achieved an average weight of 130.6 mg $plant^{-1}$ whereas strains that did not identify with the inoculant strains yielded only 64.9 mg $plant^{-1}$.

- When slow-growing rhizobia from *Desmodium canadense* were recovered from soil and characterized, only 13.7% of the strains identified with the inoculant strains used. Most of these were not those intended for *Desmodium* but corresponded to strains for *Chamaecrista fasciculata,* the legume species most abundant in the first season after planting. Further, several of the strains recovered from the field appeared better in nodulation and nitrogen fixation than were the inoculant strains tested. Additional studies to improve inoculant response to slow-growing rhizobia, to enhance inoculant strain nodule occupancy with *Desmodium,* and to better determine the importance of early establishment of the different legumes are still needed.

Although differences in performance between the granular clay, granular peat and wheat-inoculated seedings were not marked, with all superior in performance to the uninoculated and seed inoculated treatments, and each having some points of advantage, our preference based on uniformity of response and convenience of use is for the granular peat-based inoculation treatment.

Levels of nitrogen fertilization used with seed blankets appear too high for any realistic expectation that we can inoculate and produce nodules in these blankets before

CHAPTER 1: Introduction

Prior to settlement, there were more than 18 million acres of prairie in Minnesota; today less than 0.5% of that remains, much of it in fragmented pockets of vegetation and in various stages of degradation. Loss or deterioration of prairie raises concerns of genetic and soil erosion, and of carbon loss and consequences for global warming. This has spurred efforts at reconstruction, with the Minnesota Department of Transportation (MNDOT) establishing native grassland areas alongside roadways, seeding areas around rest stops with prairie plants, and undertaking extensive restoration with native plants for wetland mitigation. Contributing to the decision to re-establish prairie areas is that while they are visually attractive, only limited attention is needed post-establishment.

Nitrogen is a limited nutrient in many natural environments (Seastedt and Knapp, 1993), with nitrogen fixation critical for plant development and the maintenance of diversity. At the other extreme, over fertilization with N or N deposition can reduce the diversity of slower growing plant species in such systems, and favor invasion (Weiss, 1999; Baer et al., 2003; Blumenthal et al., 2003; Suguenza et al., 2006; Britton et al., 2007; Gendron and Wilson, 2007**)**. Because of their ability to nodulate and fix nitrogen in symbiosis with soil bacteria known commonly as rhizobia, and to meter fixed N to associated non-nitrogen fixing species, legumes have long been recognized for their contribution of nitrogen to natural ecosystems (Fred et al., 1932: Wild; 1988; Spehn et al., 2002; van der Heijden et al., 2006). For this reason, a number of indigenous legumes are included in MNDOT roadside revegetation and wetland reconstruction plant mixes. These include *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea purpurea* and *D. candida, Desmodium canadense* and *Lespedeza capitata.* Unfortunately, not all the areas used for revegetation contain the rhizobia needed for symbiosis with these legumes (Kindscher and Tieszen, 1998; Larson and Sieman, 1998). In an agricultural situation, it is easy to obtain commercial preparations of the appropriate rhizobia, and to inoculate seed or soil in such a way that the inoculated rhizobia still persist and dominate in the soil even 10-15 years afterward. Rates of N_2 fixation for agricultural species vary with legume and with the success of inoculation, but may be as much as 450 kg N ha^{-1} $year^{-1}$ (Unkovich and Pate, 2000). Unfortunately, and while nowhere near this level of nitrogen fixation is needed in the prairie situation, successful inoculation of prairies or of prairie legumes with an appropriate strain(s) of rhizobia presents problems not encountered in agricultural situations. Thus:

● Information on the *Rhizobium* requirements of the legumes used in mid-western prairie revegetation is limited, with little advance in our knowledge in recent years. Most information dates to Fred, Baldwin and McCoy (1932), Bushnell and Sarles (1937); Shave and Pengra (1974) and Falken and Pengra (1976), and many of the organisms collected in these early studies have now been lost.

● Seeding of new prairie areas commonly takes place toward the end of the growing season with host and rhizobia exposed near the surface over the subsequent winter period

● In the agricultural situation the norm is to seed a single plant species, often at densities exceeding 100,000 plants ha^{-1}. These commonly are inoculated at a rate of 10^3 to 10^6 rhizobia per seed (Graham, 2005a, b) with the result that as much as 25 kg ha^{-1} of nodules may be produced. When these senesce at the end of the growing season,upwards of 10^9 rhizobia g^{-1} of nodule tissue may be released back into the soil (McDermott et al., 1987), acting as an inoculant for subsequent crops of that legume, and in many circumstances meaning that there will be only limited response to inoculation in subsequent years. This is in contrast to prairie restoration,

where more than 40 different plant species including 10 legumes may be included in the prairie mix; the rate of seeding of a given legume may be relatively low; and the number of rhizobia that can be applied to small-seeded legume species through seed inoculation is limited. This further limits rhizobial survival over winter in the soil, can lead to spatial differences in the distribution of rhizobia, and can affect subsequent nodulation and nitrogen fixation.

● Several of the legumes used in restoration settings have distinct germination requirements, and are dependent on physical or chemical seed scarification or moist/cold stratification (Prairie Moon Nursery, 2008) before they can break dormancy. Even when these treatments have been applied, seed germination may still be delayed and irregular.

● Because of the limited market size many inoculant companies will not manufacture prairie legume inoculants, or do the research needed to maintain a product equal in quality with the inoculants for plants such as soybean, clover or alfalfa.

●. Multiple legumes are seeded in the prairie situation, with several of these able to nodulate with different inoculant rhizobia, but not necessarily to fix nitrogen during symbiosis with this range of organisms. Thus the average rate of fixation per plant under prairie conditions is likely to be much less than in the comparable agricultural situation.

The main goal of this project was to compare different methods of inoculation proposed for use with prairie legumes, and to follow the effects of such inoculation on legume composition and prairie development over time. We wanted also to determine the influence of inoculation on soil properties, particularly those related to soil quality, and to follow the rate of recovery of the inoculant rhizobia from soil and rhizosphere. The use of winter wheat as a surrogate host for rhizobia, allowing greater numbers of rhizobia to be applied, and providing rhizosphere habitat for the rhizobia until the germination of their legume hosts, is emphasized in this study. This approach has been suggested before for agricultural situations (Diatloff, 1969) but rarely made sense where there was no impediment to nodulation of the subsequently seeded legume. Earlier studies in the *Rhizobium* Research Laboratory (T. Doan and P. Graham, unpublished) suggested that this approach could have value in the prairie environment given the unique challenges to rhizobial survival and establishment.

The information in this report is organized into seven chapters. Chapters 2 and 3 review inoculation, nodulation and nitrogen fixation from a prairie perspective, and the methods used in their study, respectively. Chapter 4 describes initial studies to contrast a range of winter wheat lines and other cereals for use as surrogate hosts in prairie inoculation, and Chapter 5 the establishment of inoculated prairies at Becker, MN, using different inoculation techniques. In reporting on this experiment we also consider prairie and legume establishment in relation to inoculation, and plant growth and development, nodulation and nitrogen fixation in the initial three years after establishment. Chapter 6 examines the possibility of inoculating prairie legumes used in seed blankets, and the problems of N fertilizer used to improve the growth of grasses in such blankets, and its inhibitory effects on legume nodulation and nitrogen fixation. Chapter 7 summarizes the conclusions of this study, and the research still needed to promote prairie establishment, function and sustainability.

CHAPTER 2: Literature review: The inoculation of legumes in revegetation and restoration settings

2.1. History of the Northern American prairie

The North American prairie biome once occupied the Great Plains from Canada to the Mexican border, and from the foothills of the Rocky Mountains to western Indiana and Wisconsin, covering approximately 162 million ha. Risser et al. (1981) divided this region into three main areas; the tallgrass prairie extending from Canada and Minnesota south to Texas, and covering 91 million ha (Kunkel and Changnon, 2003), the mixed grass prairie from Canada and eastern North Dakota south to Texas (62.8 million ha, USGS, 2004), and the shortgrass plains reaching from Montana to western Texas and New Mexico (11 million ha, USGS, 2004) (see Figure 2.1). In Minnesota the tallgrass prairie occupied nearly 7.3 million ha in the western third of the state (Djupstrom, 1988). At its peak, and aided by periodic fire and grazing (Weaver et al., 1996), it is estimated that this biome supported perhaps 1000 plant species (Kindscher and Wells, 1995).

Figure 2.1. Distribution of the Tallgrass, Mixed-grass and Shortgrass prairie areas of the United States (after Risser et al., 1981).

Changnon et al. (2002) and Kunkel and Changnon (2003) suggest that the boundaries of the tallgrass prairie were defined by four climatic extremes:

- 90% more years of severe drought than forested areas to the north and south:
- frequent very dry, cold seasons with more fires triggered by lightning, and
- more frequent low evapotranspiration rates toward the west of the region.

The mixed grass prairie in the central third of the Great Plains was continental in climate with seasonal moisture and extreme temperatures, and annual precipitation declining toward the west (Bryson and Hare, 1974), while the shortgrass prairie was a characterized by water stress and with relatively few forb species (Daubenmire, 1978; Weaver, 1983).

Tallgrass prairie vegetation was dominated by C_4 grasses including big bluestem, switch grass, Indian grass, and rough dropseed, but with mid- and short-statured C_4 grasses including little bluestem, sideoats, hairy and blue grama, western wheatgrass, and buffalo grass also important (Steinauer and Collins, 1996). Bragg and Steuter (1996) distinguished three vegetationally different areas of mixed prairie:

- the northern mixed prairie with western wheatgrass, bluestem, blue grama, needle-and-thread, green needlegrass, and porcupine grass

● the Sandhills prairie dominated by warm-season grasses including prairie sandreed, sand bluestem, big bluestem, little bluestem, blue grama, hairy grama, needle-and-thread, and sand dropseed
● the southern mixed prairie with blue grama, sideoats grama, western wheatgrass, little bluestem, junegrass, green needlegrass, porcupine grass, Kentucky bluegrass, tall dropseed, and Canada wild rye dominant

Grasses in the shortgrass prairie included the warm-season species blue grama and buffalo grass, with thread leafed sedge, junegrass, and hairy grama more common in the wetter years (Weaver et al., 1996).

Forbs in the major prairie areas commonly accounted for only 10 to 25% of the ground cover (Kindscher and Tieszen, 1998; Briggs and Knapp, 2001), but contributed much more significantly to prairie diversity. They included legumes critical to the supply of nitrogen following fire or grazing (Towne and Knapp, 1996) including species of *Amorpha, Amphicarpaea, Apios, Astragalus, Baptisia, Cassia, Chamaecrista, Crotalaria, Dalea, Desmodium, Desmanthus, Gleditsia, Glycyrrhiza, Gymnocladus, Hedysarum, Lathyrus, Lespedeza, Lupinus, Oxytropis, Psoralea, Robinia, Shrankia, Strophostyles, Tephrosia, Thermopsis,* and *Vicia* (Bushnell and Sarles, 1937; Whitman and Stevens, 1952**;** Shave and Pengra, 1974; Betz and Lamp, 1989; Ownbey and Morley, 1991; De Haan et al., 2003).

The arrival of European settlers, and passage of both the United States Homestead Act of 1862 and the Canada Dominion Land Act of 1872, led to a dramatic breakup of the prairie region (Curtis, 1959; Samson and Knopf.1994) and its conversion to agriculture. Samson and Knopf (1994) suggest that the areas of tallgrass, mixed grass, and shortgrass prairies have declined by 99.9, 61, and 85.5, respectively since this time. Samson and Knopf (1996) describe this as the greatest loss of any ecosystem in North America. Remnants of the tallgrass prairie are now limited to small, highly fragmented areas found alongside roads and rail tracks, or in pockets of land inaccessible to agriculture. They are often highly degraded and most can no longer be managed by periodic burning and grazing. As such landscapes are fragmented and individual parcels decrease in size they lose diversity and vigor (Young and Clarke, 2000), Thus Samson and Knopf (1994) note that 55 of the up to 350 grassland species documented to occur in this region (Weaver and Florey, 1934; Risser et al., 1981; Howe, 1994a) are now threatened with extinction, or are endangered. Similarly, Leach and Givnish (1996) showed an 8 to 60% loss in plant species across 54 prairie remnants in Wisconsin, with an average decline in number of legume species of 23.7%. The decline among legumes was not primarily due to low persistence, but rather to limited recruitment.

Land described as the heartland of America, but now in agriculture for many years, has declined in quality, and is increasingly difficult to manage for maximum productivity (Cassman, 1999). Brye and Pirani (2005) compared soil quality traits in five prairies from the Grand Prairie region of Arkansas with adjacent agricultural lands noting a 17 to 52% decline in sand content, soil organic matter, total N and total C and extractable S and Mn with the transition from prairie to tilled soil, but a more than 50% increase in % clay, and extractable P, K, Ca, Mg, Na. Cu, Zn and Fe. Somewhat comparable data is provided by Reeder et al. (1998), Knops and Tilman (2000) and McLachlan and Knispel (2005)

Samson and Knopf (1994) make the case for prairie conservation and restoration in North America, and a number of relatively recent initiatives now support that goal. These include the USDA Conservation Reserve Program (CRP) with more than 4.86 million ha under various types of conservation practice in 2006 (http://www.fsa.usda.gov); the Grassland Heritage

foundation (http://www.grasslandheritage.org/prairie_directory) with collaborators and prairie projects in 12 states; the Nature Conservancy, Ducks Unlimited, and various State Departments of Natural Resources and/or Transportation. More recently prairie restoration has been explicitly invoked both in the mitigation of global warming and as a bioenergy source. Grasslands are generally a net sink of both CO_2 and CH_4 (Frank and Dugas, 2001; Frank et al., 2000; Sims and Bradford, 2001; Suyker and Verma, 2001), Mensah et al. (2003) noting that restored grasslands can sequester 0.7 Mg C ha^{-1} yr^{-1}.

Not all prairie restoration and roadside revegetation programs have been successful. Samson et al. (2004) note that CRP lands in the shortgrass prairie region experience significantly more soil erosion than observed in nearby native prairies, while numerous authors suggest that it can take many years for restored prairie areas to achieve the diversity and soil quality of the undisturbed prairie (Burke et al., 1995; Kindscher and Tieszen, 1998; Baer et al., 2000; Brye et al., 2002; Brye and Kucharik, 2003: Camill et al., 2004; Kucharik et al., 2006). Biofuel initiatives using CRP land (Fargione et al., 2008) could also reduce the number of prairie initiatives.

Legume establishment and persistence, and nitrogen management are important factors in the speed of restoration, the reason our laboratory has received support from the Minnesota Department of Transportation to study the inoculation and nitrogen (N_2) fixation of prairie legumes. This review considers some of the issues involved in improving nodulation and N_2 fixation under prairie conditions, and outlines specific areas where there is still a significant need for more information.

2.2. Contribution of legumes to prairies and other natural ecosystems

Legumes have played a role in the productivity of natural and agricultural systems since the time of the Romans, with many natural ecosystems dependent on these plants as a source of nitrogen. Legumes are almost unique among plant species in their ability to symbiose with a group of soil bacteria collectively known as rhizobia. Interaction between legume host and rhizobia results in the formation of numerous gall-like nodules on the root, or sometimes the stem of the legume, with bacteria within the nodule then able to reduce N_2 gas from the atmosphere into ammonia (Graham, 2005a). The bacteria get energy from their host for growth and N_2 fixation, and are protected from edaphic stress; the legume gains access to a form of nitrogen it could not otherwise utilize. On a global scale, Cleveland et al (1999) estimate that symbiotic N_2 fixation in natural ecosystems, undertaken principally though the symbiosis between legumes and root-nodule bacteria, contributes ~ 195 +/- 90 TG N yr^{-1}, with annual rates of N_2 fixation ranging from 0.1 to 10 kg ha^{-1}. While it could not be expected that legumes in a prairie environment fixed N_2 at a rate equivalent to the 137.8 kg ha^{-1} $annum^{-1}$ average reported for nine temperate pasture legumes by Peoples et al. (1995), the rates cited by Cleveland et al. seem low for much of the prairie environment. Unfortunately, the data available is very limited. Brye et al. (2002) noted soil N accumulation in a 19-24 year old tallgrass prairie of 173 kg N ha^{-1} $year^{-1}$, with 77% of that unaccounted for in N budgets and presumably due to N_2 fixation. Total N flow in the tallgrass prairie was estimated by Risser and Parton (1982) to be 244 kg ha^{-1} $annum^{-1}$, but this figure did not specifically consider N_2 fixation. Woodmansee (1978) reported fixation in one of three seasons as 25 kg ha^{-1}. Additional indirect indicators suggesting that N_2 fixation in managed prairies could be significant include:

● direct response to inoculation with *Rhizobium* under field conditions obtained with *Dalea purpurea, Desmodium canadense* and *Desmanthus illinoensis* (Tlusty et al., 2004; Beyhaut et al., 2006a).
● evidence from ^{15}N isotope-dilution (Hiers et al., 2003) and natural-abundance studies (Graham, 2004) from which it appeared that while N_2 fixation in *Rhynchosia* and *Astragalus canadensis* could be limited, species of *Dalea, Lespedeza, Desmodium* and *Centosema* were meeting 70 to 96% of their nitrogen needs through symbiosis.
● significant accumulation of microbial biomass N in five-year old prairie restorations on a sandy soil at Becker MN, with only 10-13 ppm soil N, even though the plots were raked annually and the debris removed (Graham et al., 2004).
● marked responses to inoculation in growth chamber and glasshouse studies with *Astragalus canadensis, Chamaecrista fasciculata, Dalea purpurea, Desmodium canadense* and *Lespedeza capitata* (Tlusty et al., 2004).
● number of nodules and acetylene reduction rates in 13-week old *Chamaecrista fasciculata* plants as high as 80 nodules plant^{-1} and 80 μ moles C_2H_4 produced plant^{-1} day^{-1} (Becker and Crockett, 1976). The slower-growing *Lespedeza capitata* also exhibited active nodule development and N_2 (C_2H_2) reduction, with plant and nodule weight highly correlated. Similar data for a wider range of host species is given by Lofton (1976).
● prairies in which legumes were not seeded or had failed to establish were reported as limited in development, and functioned poorly even 35 years after establishment (Kindscher and Tieszen, 1998). Larson and Siemann (1998) noted that the limited growth of *Lespedeza capitata* in N-poor grasslands could have been due to lack of suitable rhizobia.
● two legumes were among only four species that over yielded under increased diversity in soils where N was a limiting nutrient (Lambers et al., 2004). Under- yielding species in this study benefited from interaction with legumes, enabling them to persist. Piper (1995) also noted that sites with the lowest soil N consistently supported greatest legume biomass.

This data notwithstanding, there is still need for more detailed quantification of rates of N_2 fixation in the prairie ecosystem.

2.3. The process of nodulation and N_2 fixation

Root-nodule bacteria can gain entry to their hosts through root-hair penetration and infection-thread formation; via wounds or sites of lateral-root emergence; or by penetration of root primordia found on the stem of plants such as *Sesbania* and *Neptunia* (stem nodulation) (Hirsch, 1992; Boogerd and van Rossum, 1997; Boivin et al., 1997). The same rhizobial strain may gain entry to one legume via root hair infection, and to another via sites of lateral root emergence. Though root-hair penetration is generally considered the most common mechanism of infection, studies are needed to confirm that this is also true for prairie legumes.

When rhizobia come in contact with immature root hairs of a compatible host, they aggregate at the root hair tip, and within minutes attach to the root hair surface. Deformation and curling of the root hair follows, with the root-hair cell wall degraded at the point of infection to permit rhizobial penetration (Fahraeus, 1957; Sahlman and Fahraeus, 1963). Rhizobia never really gain intracellular access to their host. As they penetrate the root-hair cell wall fresh plant-derived material is deposited around them, and as they move down the root hair in the direction of the cortex, they remain enclosed within a tubular, plant-derived infection thread (Gage, 2004). In the more primitive (eg *Bradyrhizobium/ Chamaecrista*) symbioses, rhizobia may never be released from the infection thread (Naisbitt et al., 1992; Sprent, 2008), Bryan et al. (1996) even

reporting infection thread formation and apparent bacteroids in the roots of non-nodulated *Gleditsia triacanthos.* Rhizobia remain enclosed within a membrane when released into cells of the host root cortex. This serves to limit host recognition and the initiation of defense responses (Gage, 2004).

These initial steps in infection are accompanied by molecular signaling between host and rhizobia (Long, 1996; Schultze and Kondorosi, 1998), with some 50 rhizobial genes now thought to be involved in the nodulation process. Organisms living saprophytically in soil do not express the majority of these genes, but only turn them on in the presence of a suitable host. The triggers for this expression are specific flavonoid compounds excreted from the legume root, and recognized by the rhizobia. Nod-gene expression results in production of an extremely powerful plant hormone by the rhizobia. This lipochitooligosaccharide or nod-factor conditions the root for the formation of the nodule. Legumes differ in the spectrum of flavonoids each produces, and rhizobia in the number, structure and the side chains of their nod-factors, with both flavonoid and nod-factor composition affecting the range of host plants nodulated (Downie, 1998). The flavonoids produced by different species of prairie legume have not been studied; the only prairie rhizobia for which nod-factor composition has been determined are *R.gallicum* and *R.giardinii* (Soria-Diaz et al., 2003, 2006).

Stimulation of plant cell division and cell enlargement in the root cortex result in the formation of a visible nodule, the shape of which is determined by the host (Dart, 1977), and regulated by the pattern of cortical cell division following infection. Thus *Dalea* and *Chamaecrista* nodules (like those of alfalfa) are elongate, and increase in length over the course of the growing season, whereas those of *Desmodium* are spherical in shape and limited in the size they can achieve (see Sprent (2008) for a more detailed description of differences in nodule shape). Nodules that are actively fixing N_2 (referred to as effective nodules) will usually have a central region in which host cells are packed with rhizobia, and that is pink or red in color due to the presence of leghemoglobin, a compound needed to regulate oxygen transfer within the nodule. Ineffective nodules will usually be white or green in color. Nodule decomposition at the end of the growing season releases rhizobia from the nodule back into the soil, where they live saprophytically until the next growing season. A major difference between determinate and indeterminate nodules (to date only studied with agricultural plant species) is that the percentage of released bacteria that are viable is very much greater for the determinate than for the indeterminate nodule (Sutton, 1983; McDermott et al., 1987). In the agricultural situation this difference is not likely to be critical. Numbers of rhizobia recovered from inoculated field soils can vary from 3.9×10^4 rhizobia g^{-1} soil early in the season, to 7.5×10^5 g^{-1} soil following nodule senescence (Hely et al., 1957; Ellis et al., 1984), but will usually decline significantly in the period without crops. In the prairie, with lesser numbers of legumes present, this difference in % bacteroid viability could be of importance, and exacerbated by predation of protozoa and nematodes on the rhizobia released.

Nodule number $plant^{-1}$ is influenced by the host genotype, by the number and efficiency of the inoculant rhizobia, and by nutritional and soil stresses. It can vary from very few to more than 300 nodules $plant^{-1}$, with nodule number and nodule size usually inversely related (Nutman, 1967). Because of this, nodule mass $plant^{-1}$ measured several times throughout the growing season is probably the best simple indicator of nodulation success. However, recovery of nodules from deep-rooted prairie legumes can be problematic.

Edaphic factors constraining legume production and nitrogen fixation in the prairie are similar to those reported for cultivated legumes (Graham and Vance, 2000; O'Hara, 2001;

Slattery et al., 2001; Graham et al., 2003). Of particular concern are N deposition, P supply and drought.

Spehn et al. (2002) found that N_2 fixation by legumes was the primary functional trait of species influencing nitrogen and biomass accumulation in planted experimental grassland sites in Europe. However, this contribution to early successional development does not necessarily carry over into later growth (Pearson and Vitousek, 2001; Rastetter et al., 2001; Vitousek et al, 2002). Factors affecting symbiotic N_2 fixation in the later stages of prairie restoration are likely to include a build up in the pool of soil N, the low tolerance of legumes to shading, and the sensitivity of N_2 fixation to limited availability of P, K, and micronutrients (Vitousek et al., 2002). Nitrogen fertilization, deposition, or build up of N in soil clearly favors faster-growing species and limits diversity (Turner et al., 1997). Though the direct effects of N fertilization or N accumulation in soil on nodulation and N_2 fixation of prairie legumes has not been studied, enhanced nitrogen availability inhibits nodule development and function in all other legumes so far studied (Dean and Clark, 1980; Eardly et al., 1985; Gan et al., 1997). van Kessel and Hartley (2000) considered increased utilization of N-fertilizers world wide a prominent factor in the decline in N_2 fixation by soybean in 362 inoculation trials carried out over the period between 1970 and 1995 (-0.7% NDFF ha^{-1} yr^{-1}). Immobilization of C, N, P and S within root tissue and soil organic matter fractions can result in lowered P availability, and a decline in N_2 fixation (Vitousek and Field, 1999; Niklaus and Korner, 2004). Grunzweig and Korner (2003) noted legume response to CO_2 enrichment substantially increased following P fertilization. It is an interesting anomaly that Wilson and Hartnett (1998) have reported the productivity of prairie legumes negatively correlated with mycorrhizal infection. One explanation for this could be nutrient loss to other plants through hyphal connections.

2.4. Legumes used in prairie restoration, CRP, etc. and their *Rhizobium* requirements

Native legumes recommended for roadside use in the different states of the USA are listed by Harper-Lore and Wilson (1999). A similar list is available online at (http://www.fhwa.dot.gov/environment/rdsduse/index.htm). Legumes used on roadsides and in CPR lands may also include non-natives such as Medicago *sativa, Trifolium hybridum, T.pratense,* and *T. repens,* (MN/DOT seeding manual, 2003). Excellent images of prairie legumes from mid-western prairies are available at http://www.inhs.uiuc.edu/~kenr/prairieplants.html). Differences in the rhizobia associated with each legume species, and the sometimes limited availability of these organisms in soils that have been in agricultural production for many years (Larson and Siemann, 1998) should require the use of rhizobial inoculants in revegetation and restoration projects.

Given the complex signaling involved in nodule formation (see pages 6-9), specificity in the interaction between legumes and rhizobia is to be expected. This can be evident at a number of levels:

- Many legumes, especially those of the Caesalpinioideae never form nodules. While the number of studies undertaken to date is limited, non-nodulated prairie legumes could include *Cassia* species (Whitty et al., 1994), *Gleditsia triacanthos* and *Schrankia* spp. Note however, that Pengra (1976) has reported nodulation of *Psoralea.*
- Each rhizobial isolate has the ability to nodulate some but not all legumes.

Legume species nodulated by the same rhizobia are often considered as belonging to a particular cross-inoculation group, with further subdivision in some cases into effectiveness subgroups differing in their ability to fix N_2 with particular rhizobia (Burton, 1967);

- Different accessions of the same plant species can vary in the level of N_2 fixation achieved (Graham et al., 2005a); and
- Mutations in host or rhizobia can lead to loss of nodule forming ability.

Ability to nodulate certain legumes and not others was for many years the major determinant of species among the root nodule bacteria (Fred, Baldwin and McCoy, 1932). It is still important in the recovery of these organisms from soil, strain identification (Graham et al., 1991), and in selecting strains for use as inoculants. Twelve genera and close to 50 species of root-nodule bacteria have now been described (Willems, 2006; Graham, 2008a), but the great majority of these are not known to nodulate any prairie legume. These species include a number of ά and β proteobacteria, only recently described as having the ability to nodulate legumes (Moulin et al., 2001; Chen et al., 2003), and still largely unstudied. These are not the dominant root-nodule bacteria associated with prairie legumes but have been recovered from the nodules of *Lupinus* and *Crotalaria* (*Sy* et al., 2001; Trujillo et al., 2005)**.** Genus and species designations and specific inoculant strain recommendations for the legumes used in restoration or roadside plantings in Minnesota are shown in Table 2.1. Note:

● Many studies on the taxonomy of rhizobia infecting legume genera found in mid-west prairies were done in China or Europe with host species important to those regions. (e.g. *Astragalus* and *Mesorhizobium huakuii* [Chen et al., 1991]; *Lespedeza* and *Bradyrhizobium yuanmingense* [Yao et al., 2002]). There is no guarantee that the same microsymbionts will be recovered from these legumes in the American prairie environment. Both beans and soybeans provide examples of this point (Graham, 2008a). Thus in the center of origin of *Phaseolus vulgaris, Rhizobium etli* is dominant, whereas in other, newer centers of production of this crop 4-5 other species may occur, and even dominate where soil conditions are acid. For soybean, *B. japonicum, B.elkanii* and *R. fredii* all occur widely in the soybean fields of China and Japan, whereas *R.fredii* is found only infrequently in similar fields of the USA and Brazil. We still do not know the genus or species of some of the prairie legume rhizobia.

● Not all rhizobia have been assigned specific names. Where only the genus of bacteria for a given legume has been identified, it is common to refer to it by the host from which it was derived, eg. *Bradyrhizobium* spp. (*Desmodium*)

● There are some prairie legumes (eg. *Psoralea esculenta*) for which no specific rhizobia have yet been identified.

Table 2.1. Legumes used in roadside and prairie revegetation, their putative rhizobia, and recommended inoculant rhizobia where known.

Legume	Rhizobia recovered	Inoculant recommendation and source
Amorpha	*M. amorphae*[1]	Smith et al. (1988)*
Amphicarpaea	*Bradyrhizobium spp*[2]	Smith et al. (1988)*
Apios	*Bradyrhizobium spp*[3]	
Astragalus	*M.huakuii*[4], *M.septentrionale*[5] *M.temperatum*[6], *M.loti*[7], *R.loessense*[8]	Smith et al. (1988)* 6355 (Tlusty et al., 2004)**
Baptisia	*Not known*	Smith et al. (1988)*
Cassia	Not nodulated?	
Chamaecrista	*Bradyrhizobium spp*[9]	EL (Smith et al.,1988)** 6404/6437 (Tlusty et al., 2004)**
Crotalaria	*Bradyrhizobium spp*[10]	EL (Smith et al.,1988)**
Dalea	*R.etli*[11], *R.gallicum*[11], *M.huakuii*[11], *M.amorphae*[11]	M,F (Smith et al., 1988)** 6808, 7205 (Tlusty et al., 2004)**
Desmodium	*Bradyrhizobium spp*[3]	EL (Smith et al., 1988)** 6437, 6617 (Tlusty et al., 2004)**
Desmanthus	*R.giardinii*[12]	Smith et al., (1988)* 6029 (Beyhaut et al., 2006a)**
Gleditsia	Not known	
Glycyrrhiza	*M.tianshanense*[13]	Smith et al. (1988)*
Gymnocladus	*Not known*	
Hedysarum	*R.sullae*[14]	Smith et al. (1988)*
Lathyrus	*R.leguminosarum* bv *viciae*[15]	C (Smith et al., 1988)*
Lespedeza	*B.yuanmingense*[16], *R.loessense*[17]	EL (Smith et al., 1988)** 6513, 6564 (Tlusty et al., 2004)**
Lupinus	*B.canariense*[18]	H (Smith et al., 2004)* 6003 (WU425) RRL***
Oxytropis	*R.gallicum*[19], *Mesorhizobium*[19]	
Psoralea	*Not known*	
Robinia	*M.amorphae*[20], *M.loti*[20], *M.huakuii*[20] *R.leguminosarum*[20], *R.tropici*[20]	Smith et al. (1988)*
Shrankia	Not nodulated?	
Strophostyles	Not known	Smith et al. (1988)*
Tephrosia	*Bradyrhizobium spp*[21]	Smith et al. (1988)*
Thermopsis	*Not known*	
Vicia	*R.leguminosarum* bv *viciae*[22]	C (Smith et al., 1988)**

[1.] Wang et al. (1999); [2.] Marr et al. (1997); [3.] Parker (1999); [4.] Chen et al. (1991); [5.6.] Gao et al. (2004); [7.] Laguerre et al. (1997); [8.] Wei et al. (2003)

[9.10.] P.Graham pers. comm. [11.] Tlusty et al. (2005); [12] Beyhaut et al (2006a,b);
[13.] Chen et al. (1995); [14.] Squartini et al. (2002); [15.] Mutch and Young (2004);
[16.] Yao et al. (2002) [17.] Wei et al. (2003) [18.] Stepkowski et al. (2005);
[19.] Laguerre et al. (1997); [20.] Ulrich and Zaspel (2000), [21] Yates et al., 2004;
[22] Mutch and Young (2004)
* Requires an inoculant specific to the particular genus of legume, or sometimes even to specific species within that genus
** Numbers or letters refer to the specific strains used in the inoculant. Commonly such inoculants can be used with a wider range of legumes, for example with most species of *Lathyrus, Vicia, Pisum and Lens*
*** Rhizobium Research Laboratory (http://www.Rhizobium.umn.edu)

The host/*Rhizobium* inoculant listing given above makes the selection of a rhizobial strain for a specific legume seem much more clear cut than is actually the case, with much work still needed to resolve issues in the recommendation of specific strains. Many of the strains listed as being of inoculant quality have yet to be tested for the full range of traits required of inoculant-quality rhizobia (see pages 12-13). Further, interrelationships between specific prairie legumes and the rhizobia that they may encounter in the field are not well defined. Problems include unexpected specificities in nodulation among legume hosts belonging to the same genus and their rhizobia, as well as promiscuity in nodulation between hosts and rhizobia from different cross-inoculation groups. Early studies of the root nodule bacteria (reviewed by Fred et al. (1932)) assumed that rhizobia from any legume in a specific cross inoculation group would nodulate all other legumes in that group. Numerous exceptions to this belief have since been reported (Wilson, 1944), and seem particularly common between prairie legumes and their rhizobia. We have recovered rhizobia with the 16S rRNA gene sequence of the highly specific bean microsymbiont *R.etli* from both *Dalea spp* and *Desmanthus illinoensis,* and have shown that these organisms also infect *Phaseolus, Leucaena, Macroptilium* and *Onobrychis* (Graham et al., 2004; Tlusty et al. 2005; Beyhaut et al., 2006b; Martir et al., 2007).This is a much wider host range than that of traditional bean *Rhizobium,* and suggests that transfer of symbiotic genes between different rhizobia in this environment could be important. As bean production in the area where these studies were undertaken is limited, we have also hypothesized that *R.etli* in this region could have been introduced as an endophyte of corn (Gutierrez-Zamora and Martinez-Romero, 2001; Rosenblueth and Martinez-Romero, 2004). Nandasena et al. (2006, 2007) have noted the transfer of a symbiotic island from the microsymbiont for *Biserrula pelecinus* to other soil organisms within six years of introduction of this host/*Rhizobium* combination, with resultant decline in symbiotic performance. It is noteworthy that the microsymbiont for *Biserrula* is *Mesorhizobium ciceri* bv *biserrulae,* and that isolates of *M.huakuii* and *M.amorphae* have also been recovered from *Dalea* (Tlusty et al., 2005). In contrast, unexpected specificity in nodulation has been evident with the rhizobia of both *Dalea* and *Desmanthus.* With the former, strain UMR 6815 was highly effective in symbiosis with all perennial *Dalea* species tested, but failed to nodulate the annual *D. leporina* (Tlusty et al., 2004, 2005), while with *Desmanthus illinoensis* none of the four inoculant quality strains identified by Beyhaut et al. (2006a) also nodulated *D. virgatus*, and all four were ineffective on *Phaseolus vulgaris.* Burton (1967) tested nine strains of slow-growing rhizobia belonging to the cowpea cross inoculation group with cowpea and with three species of *Lespedeza.* All strains were active in N_2 fixation with cowpea, but no strain was highly effective in symbiosis with all three *Lespedeza* species tested. We have

had similar problems identifying broadly effective bradyrhizobia for use with *Chamaecrista, Desmodium* and *Lespedeza* in Minnesota prairie mixes. Additional studies are needed to clarify cross inoculation among prairie legumes, and to determine the extent of genetic interchange and its effects on host plant range.

Legume collection from different regions of the world is often complicated by the need to match collections of the host with those of suitable rhizobia (Lie et al., 1987; Date, 1982). With local ecotypes commonly required in restoration seed mixes (MNDOT seeding manual, 2003; Erickson and Navarett-Tindall, 2004), the possibility also exists that rhizobial requirement might also need to vary with region.

2.5. Legume inoculants: strains, formulations and uses

The legume inoculant industry had its beginnings in 1895 using laboratory grown cultures produced on the surface of a gelatin based medium containing sugar and legume extract (Burton, 1967). Seventeen different inoculants were produced, one for each important legume crop, and all failed to live up to expectation. Early problems are reviewed by Fred et al. (1932), and necessitated better definition of cross-inoculation group boundaries; regulatory testing of legume inoculants at the state or national level (for example Rennie and Hynes, 1993**,** Bullard et al., 2005), the development of submerged culture methods, and the identification of more effective inoculant carriers and methods of inoculation, before significant progress could be made (Burton, 1967). Today, there are perhaps 4 major and a number of smaller producers active in North America, and most agriculturally important crop species can be effectively, if not ideally inoculated. That there is still room for improvement is evident in two recent quotations. "We enter the era of biotechnology knowing more and more about the mechanisms of N_2 fixation at the gene level, but except for some manufacturers in developed countries …still lacking good quality and reliable inoculants." (Catroux et al., 2001) and "Despite nearly one hundred years of experience it is unfortunately true that most of the inoculant produced in the world today is of relatively poor quality……and even good quality inoculants are often not used to the best advantage " (Brockwell and Bottomley, 1995).

Current inoculant companies emphasize major crop legumes, and rarely advertise inoculant strains for the smaller volume niche legumes or for restoration activities. They may have other inoculant strains that are available for research purposes, or for sale on request. Support for field studies with the lesser legumes also appears to be lacking. Some of the major commercial inoculants, for example EMD Biosciences strain EL or the Becker Underwood cowpea inoculant could serve as inoculants for *Chamaecrista fasciculata, Crotalaria sagitallis, Desmodium canadense,* and *Lespedeza capitata*, but were not selected for their response with these species, and may not work optimally with each one. The *Rhizobium* Research Laboratory (http://www.Rhizobium.umn.edu) produces limited quantities of prairie legume inoculant, only because we know of the difficulty in getting inoculant quality strains for the legumes with which we work. Our strains were each selected following the evaluation of 75-100 isolates per species (Tlusty et al., 2004), but have not been tested for anything like the full range of attributes desirable in an inoculant-quality rhizobia. These were defined by Thompson (1991) as:

- able to form highly effective nodules with all commonly used varieties of the legume species for which it is recommended,
- competitive in nodule formation and persistent in the soil,
- able to tolerate soil environmental stresses such as pH and temperature,
- have good growth in simple, inexpensive culture media,

- be genetically stable and not subject to mutation,
- able to survive well on the seed prior to seed germination, and
- have the ability to persist in soil between crops.

The importance of just one of these traits is evident in the work of Catroux (2005) who showed that the growth temperature used in strain evaluations markedly affected which strains were selected.

Inoculants for the major species used in agriculture are available in a number of different formulations. These include sterile and non-sterile peat; different granular clay and peat formulations, and for some species only, liquid formulations that permit inoculation and fungicide treatment of seeds well in advance of seeding (Deaker et al., 2004; Bullard et al., 2005; Herridge, 2008). As an example of the care needed with liquid formulations, it is our experience that the commonly available liquid formulations work well with soybean rhizobia but poorly with the rhizobia from beans. We are currently evaluating how to modify these formulations so they will have wider application. To be effective an inoculant carrier must:

- have high water-holding capacity;
- maintain high population counts of rhizobia for a minimum period of 6 months;
- be readily availability, of low cost, and easily processed;
- be sterilizable, and
- provide good buffering capacity (Thompson, 1991)

In recent years, a number of different additives, the majority proprietary, have been used with inoculants. These include stickers that promote adhesion of the rhizobia to the seed, extenders that prolong the life of rhizobia on the seed, flavonoids to promote nodulation at low temperature (Leibovitch et al., 2001; Zhang and Smith, 2002), and protectants against osmotic and drought stress, or contact with fungicide or fertilizer (Smith, 2004), and nod-factor (Osburn et al., 2004)**.** A number of *Rhizobium* inoculants can now also contain other organisms, including a mix of rhizobia for the inoculation of multiple legumes (garden inoculants), and biocontrol or phosphate solubilizing organisms such as *Bacillus* and *Penicillium* (Rice et al., 1995). Rhizobia in broth culture, liquid formulation or in sterile peat after maturation should have counts of at least 10^9 rhizobia mL^{-1} or g^{-1} (Lupwayi et al., 2000; H.H.Keyser and P.Singleton, pers. comm.). Counts in non-sterile peat or on granules are likely to be less (Lupwayi et al., 2000; Bullard et al., 2005)**.** Properly prepared and stored, sterile-peat or liquid preparations should maintain such populations for periods of up to one year; longer storage can result in changes in the physiology and infectiveness of the inoculant organism, even though there has been only limited change in cell counts (Maurice et al., 2001).

Inoculants can be either seed or soil applied, with sterile or non sterile fine peat or liquid formulations mainly used for seed inoculation, and granular formulations used where the inoculant is to be broadcast or banded under the seed (Kyei-Boahen et al., 2002). When appropriately used, the expectation is that the number of rhizobia supplied for seed inoculation will vary with seed size, but exceed 10^3, 10^4 and 10^5 rhizobia $seed^{-1}$ for small, medium and large-seeded seeds, respectively (Lupwayi et al., 2000; Deaker et al., 2004; Bullard et al.,2005). For non-sterile peat this corresponds to 5 g peat inoculant per kg seed; lower rates are sometimes recommended for the higher count sterile peats or for liquid formulations (Somasegaran and Hoben, 1994). Granular inoculants are usually applied at the rate of 10 kg ha^{-1}, or ~ 1 g inoculant m^{-1} of row (FAO, 1984). Less commonly, rhizobia have been applied in the irrigation water, in this case supplying perhaps 10^{14} rhizobia ha^{-1} (Ciarfardini and Lombardo, 1991)**.** Quality control for such inoculant preparations can be quite variable, but is strictly regulated in countries such as

Australia, Canada, France, and Uruguay (Rennie and Hynes, 1993**;** Lupwayi et al., 2000, Bullard et al., 2005).

A number of additional carrier materials and additives including alginate and polyacrylamide beads, oils, charcoal, bagasse, milk whey and sewage sludge are reported in the literature, but have not yet been widely accepted for use with commercial inoculants. They are reviewed by Deaker et al. (2004) and Herridge (2008)

There are major differences between the inoculation of agricultural and prairie legumes. First, in contrast to the situation with the agricultural species described above, prairie legumes are often planted without inoculation, or using low quality inoculants. Most of these have been produced using non-sterile peat intended for seed inoculation, and in many cases these inoculants have been opened and subdivided at the seed dealer, without thought of possible effects of subsequent desiccation on the rhizobia. Many such inoculants must have few viable rhizobia. Second, in prairie seeding, each of the 4-6 legumes supplied will commonly constitute only 0.5 to 1.5% of the seed mix (Prairie Moon, Winona, MN); that is around 50,000 seeds per ha. Applied at conventional rates, each legume would receive only ~5 x 10^8 rhizobia ha^{-1}. This is clearly less than supplied in an agricultural setting, and is perhaps the reason we have found rhizobia concentrated in the region underneath their host plant (Graham et al., 2004). Third, where agricultural crops in the Great Plains are mostly spring planted, and have been selected for uniform and prompt germination, ensuring little delay between planting and infection by rhizobia, prairie sowings are often undertaken in the fall. Prairie seeds can have germination requirements, and in the case of the legumes are mostly hard seeded. They may need scarification in concentrated sulfuric acid, or cold/moist treatment, to break seed dormancy. The result is that even where they are appropriately inoculated, the rhizobia may have to persist in soil for 9-15 months before they can encounter and infect their host. Where stress conditions in the soil mean that most inoculant rhizobia will die between inoculation and host germination, it is an additional complication that cross inoculation between surviving rhizobia and the different legumes in the seed mix is a possibility (Graham et al., 2004; Tlusty et al 2005). Competition between inoculant and indigenous rhizobia in soil is a significant problem with agricultural plants. We don't know yet how significant it can be in prairie seedings. Research activities needed to resolve some of these problems include:

● Evaluation of granular inoculants for use with prairie legumes. These could be mixed with and broadcast with the seed, supplying more organisms ha^{-1}, and better survival.

● Evaluation of alternate inoculation strategies. These might include the inoculation of wheat or oats used as a cover crop for the prairie planting. Use of non-legume seed to deliver inoculant to the actual legume host is an old technique (Diatloff, 1969), but in most agricultural situations makes little sense. Oats and winter wheat are often used as cover crops in Spring and Fall prairie plantings, and in the case of wheat would allow some growth of cereal prior to the first frost, and establishment of *Rhizobium* in the wheat rhizosphere. Rhizobia are capable rhizosphere colonizers, and there is increasing evidence that they can act as endophytes with a number of cereals including corn, rice and wheat (Hilali et al, 2001: Gutierrez-Zamora et al., 2001; Yanni et al., 2001; Rosenblueth and Martinez-Romero, 2004). The old cultivar Roughrider has been shown to support high populations of *Dalea* rhizobia in the rhizosphere (T. Doan and P.Graham, unpublished data) but additional field studies to test the survival of different prairie inoculant strains on wheat, and their interactions on this alternate host, are still needed.

● Improvement in the response of rhizobia to cold and drought stress. Such studies could consider the importance of protectants and stickers, and how culture media modification during

inoculant production might enhance subsequent cold and desiccation tolerance. Maltose, trehalose, and glycine betaine have all been identified as osmoprotectants for rhizobia (Bushby and Marshall, 1977; Miller and Wood, 1996); Streeter (2003) showing that addition of trehalose to culture medium during fermentation enhances survival under desiccation stress. Drought stressed nodules of *Phaseolus vulgaris* have also been shown to accumulate trehalose (Zacarias et al., 2004).

● Improvements in the germination of prairie seed. Hard-seededness, and the germination of seeds over a prolonged period of time is an avoidance mechanism in natural environments where water supply is problematic, and it ensures that some seeds will survive even severe drought. It is, however, a significant problem from the perspective of inoculation. Seed scarification and cold/moist stratification are practiced by some seed producers; Lofton (1976) obtained 100% germination of *Desmanthus leptolobus* and *Neptunia lutea* by scarification in concentrated sulfuric acid for one hour, a period considerably longer than recommended by Somasegaran and Hoben (1994). Testing of the smoke-derived chemical 3 methyl-2H-furol[2,3-c]pyran-2-one (Flematti et al., 2004; van Staden et al (2006) and Nod-factor (Prithyviraj et al., 2003) as stimulants of seed germination and early growth are also warranted. Prithiviraj et al. (2003) have noted enhanced germination and early plant growth in a number of crop species, following treatment with nod-factor, but effects on the germination of prairie species has not been studied. Were such treatments able to enhance early germination of prairie species including legumes, it would be important that seed mixtures used in restoration activities contain both treated and untreated seeds to limit seed loss in case of early season drought.

2.6. Prairie restoration/ management

In the prairie before 1800, fire and grazing were critical natural management factors (Anderson, 1990, Collins, 1990), removing detritus (Hulbert, 1988; Brye et al., 2002), changing the amounts and availability of soil nutrients (Knapp and Seastedt, 1986. Ojima et al 1994; Brye et al., 2002), regulating below ground processes (Johnson and Matchett, 2001), and limiting incursion of invasive species (Briggs et al., 2005). Brye et al. (2002) list the post burn ash proportions of P, K, Ca, Mg and S as 0.35, 0.54, 1.89, 0.40 and 0.11% respectively.

Post-burning, there was consistent increase in plant growth (Engle and Bidwell, 2001). This was due in part to a reduction in shading, permitting earlier warming of the prairie soils (Ojima et al., 1994), to the added availability of some nutrients in the ash, and to the opportunities for growth of more diverse plant species. Thus while N was lost during burning, with further immobilization of N as roots decomposed (Brye et al., 2002; Ojima et al., 1994; Dell and Rice, 2005), fire favored both species with high nitrogen use efficiency, and plants able to access other sources of N. Legumes in particular were benefited, Towne and Knapp (1996) noting legume density increased from 3.0 to 8.0 stems m^2 after burning, with *Desmodium illinoense* the only legume not responding to fire.

Grazing in the prairie system has a similar, but subtly different role to fire. Diversity is enhanced, and habitat heterogeneity increased, while the dominance of C_4 grasses is lessened (Collins et al., 1998). Grazing preferences allow forbs to flourish, while substantially altering nutrient cycling processes and patterns of nutrient availability. N from urea and feces can impact N_2 fixation (Knapp et al., 1999; Menneer et al., 2004)

Fire frequency in prairie fragments and restorations is often reduced, with no or only limited opportunity for grazing. N deposition can be a further problem increasing the rate of growth of C_4 grasses, and negatively affecting diversity (Zavaleta et al., 2003; Smart et al, 2005).

Both changes affect legume growth and diversity (Baer et al., 2004), Leach and Givnish (1996) noting that legumes were among the species most affected by fire suppression. Graham et al. (2004), Collins et al. (1998) and Tix and Charvat (2005) mowed and raked prairie areas to encourage N limitation and favor plant diversity during establishment. This approach results in a high incidence of legumes in the prairie, with soil microbial N levels enhanced (Graham et al., 2004).

A number of studies have imposed N fertilization (Baer et al., 2003) or N immobilization treatments on prairies as a means to study the effect of N level on prairie diversity and function. Adding fertilizer N to newly established prairies, as is common in many restoration settings to encourage early ground cover, enhances productivity but reduces diversity (Baer et al., 2003), with consequent potential for invasion (Naeem et al., 2000; Kennedy et al., 2002) . Build up of nitrogen in prairies that are neither burned nor mowed could have a similar effect. We know of no data relevant to the prairie ecosystem but in *Acacia koa* Pearson and Vitousek (2001) found nodulation and nitrogen fixation reduced significantly in twenty year stands. N immobilization through addition of sugar or sawdust (Reever Morghan and Seastedt, 1999, Baer et al., 2003; Blumenthal et al., 2003; Prober et al., 2005) decreases overall productivity but favors diversity and limits weeds.

In this regard CRP land containing legumes and mown to provide feed or biofuel might be expected to maintain higher levels of N_2 fixation, and to select for more effective strains of rhizobia because of the constant drain on N through harvest. They would undoubtedly require P or K supplementation, and possibly the supply of other nutrients removed in the stover over time.

2.7. Conclusions

Agricultural development over much of the 162 million ha of prairie grassland in the Great Plains of the US and Canada has left behind a deeply fragmented region in which the areas of tallgrass, mixed grass and shortgrass prairies have declined by 99.9, 61.0 and 85.5% respectively. Restoration is critical for agricultural, CRP and wildlife maintenance, but for any site is a slow multi-year process. Legumes and the levels of nitrogen they can contribute are integral to this development, and where legumes have not been planted or have failed to establish, prairie development and diversity establishment will be delayed. From multiple indirect indicators it appears that nodulation and nitrogen fixation play an equally important role in legume establishment and function, but there are many holes in our knowledge of this process. Key issues for further study need to include more detailed inoculant strain evaluation, including a more careful consideration of whether all prairie legumes need to be inoculated; development of inoculant formulations and alternate inoculant technologies that will ensure the establishment of these rhizobia; in depth study of the role of N_2 fixation in prairie maintenance, and the levels of fixation achieved; and an understanding of the ecology and possible genetic interaction of the different inoculant and native rhizobia in prairie soils. Management of restoration areas to maintain diversity is also critical and also needs to be more carefully evaluated.

CHAPTER 3: Literature review: Measuring the success of inoculant rhizobia in nodulation, and the contribution of the inoculant strain(s) to nitrogen fixation and plant growth

Rhizobia constitute a highly variable proportion of the organisms in soil, but can be as much as 25% of the rhizosphere population (Graham, 2008b). Their numbers in soil are influenced by presence of a suitable host, inoculation practice, the soil physical and chemical environment, fertility level and the presence or absence of pesticides or pollutants. When a new species is introduced, or where prairie soils are returned to legumes after a significant period under another system, the soil will rarely contain an adequate number of effective rhizobia and nodulation and nitrogen fixation will be less than optimal (Scudder, 1975; Abaidoo et al., 2007). Inoculation with infective and effective rhizobia at this time will usually benefit plant establishment and productivity (Brockwell et al., 1982; Beyhaut et al., 2006a), and lead to the build up of rhizobia in the soil. A number of different parameters can be used to monitor inoculation success, to quantify improvements in nodulation and nitrogen-fixation ability, and to determine rhizobial numbers in soil or nodules, and whether or not they derive from the inoculant. Not all of these parameters are equally applicable to the agricultural and prairie situation, and in a prairie context may be difficult to use, or imprecise. In consequence, several different approaches are often needed for the effective evaluation of response to inoculation. Parameters employed to measure inoculant response, or the contribution of inoculant strains to nodulation and nitrogen fixation are reviewed by Giller (2001) and include:

3.1. Percentage of plants nodulated or nodule number per plant

Percentage of plants nodulated and nodule number per plant can vary with numbers of indigenous rhizobia in soil, with the inoculant strain(s) used and with host genotype (Nutman, 1967; Graham, 1973). They will also be affected by environment, particularly soil acidity, availability of water and temperature (Vargas and Graham, 1989; Brockwell et al., 1991) and by the effects of pollution (Lakzian et al., 2002). Each of these measurements is more likely to be of value under adverse conditions tending to significantly reduce the number of nodules formed (for example low numbers of rhizobia applied, acid soil pH, etc), or where plants are small and removing nodules for weighing and analysis can be problematic. They are a less useful measure of response under N-limited, but otherwise productive soil conditions, where plants are large and can have many hundreds of nodules, or where the infective strain is not effective in nitrogen fixation, regulation of nodulation is not imposed, and the host continues to produce numerous small ineffective nodules (Nutman, 1967). Both nodule number and nodule mass determinations require that plants are eased very carefully from soil, as many nodules can be lost during harvest. Counting or separating and weighing the nodules of deep rooted prairie legumes can be difficult, destructive of the prairie, and involve significant experimental error. Appropriate controls must also be included in such studies and establish numbers of indigenous rhizobia, rhizobia introduced as seed contaminants or by wind, and the possibility of cross contamination between plots, the most effective control being inclusion of a treatment without inoculation and that uses surface-sterilized seed.

3.2. Nodule mass per plant

Plants regulate nodule formation, limiting number of nodules formed and their size and activity when they believe they have sufficient nitrogen for their growth needs (Tanner and

Anderson, 1964; Gibson and Harper, 1985; Malik et al., 1987; Bollman and Vessey, 2006) The most obvious impact of this is under high soil N conditions where nodulation is markedly inhibited, the reason that N fertilization should not be used in association with inoculated legumes (Tanner and Anderson, 1964; Hungria et al., 2006). Commonly, where the strains used are highly effective in symbiosis, and N is limiting, fewer, larger nodules will be produced, and there will be a strong correlation between nodule mass per plant and plant growth and yield (Tlusty et al., 2004). Nodules however are not long lived. Initial nodules produced on soybean, for example, have usually undergone significant senescence by 80 days after planting, McDermott and Graham (1989) and Wolyn et al (1989) each demonstrating the importance of subsequent lateral root nodulation to nitrogen fixation by the plant post flowering. Changes in nodule mass and activity must then be a factor.

3.3. Plant Dry weight

Legume dry matter production in plants grown under N limited conditions is highly dependent on nitrogen fixation and so is nearly always very strongly correlated with nodule mass and the efficiency of the symbiosis. A significant percentage of that biomass remains underground at harvest, but in deeply rooted prairie plants is difficult to access.

3.4. Total plant nitrogen

Nitrogen accumulated by the plant through N assimilation or nitrogen fixation is used in plant growth, but tends to be diluted out by further plant growth rather than accumulate in the tissues. As a result total plant N rather than N concentration is the better indicator of nitrogen fixation under N-limiting conditions, and should contrast plants that have received inoculation from those that have not. A parameter commonly used is that of % plant N derived from fixation, van Kessel and Hartley (2000) noting that for soybean grown in the USA this figure declined from 68 to 50% between 1970 and 1995. While not detailed in the case of prairie legumes, N deposition could be expected to have had similar impact.

3.5. ^{15}N natural abundance

Nitrogen in natural environments occurs in the form of two stable isotopes ^{14}N and ^{15}N, with the former most affected by denitrification and leaching losses from soil. Thus, while ^{14}N is dominant in both soil and atmosphere, the concentration of ^{15}N is relatively greater in soil. The result is that leguminous plants able to meet at least part of their nitrogen needs through symbiosis tend to have a higher proportion of ^{14}N than ^{15}N in their tissues. This difference can be measured relative to appropriate non nitrogen-fixing controls using a stable isotope mass spectrometer coupled to a C/N autoanalyzer (Gathumbi et al., 2002) with the % of nitrogen in the plant derived from nitrogen fixation given by the equation

$$\frac{[\delta^{15}\text{N reference crop} - \delta^{15}\text{N legume}]}{[\delta^{15}\text{N reference crop} - B]} \times 100$$

where B is a measure of isotopic fractionation in the legume grown without N fertilization. While this methodology appears appropriate to legumes in the prairies, where coneflowers or grasses can be used as the non nitrogen-fixing control, there is the risk that a) nitrogen-fixing and non-fixing plants will explore different fractions of the soil and so differ in N accumulation pattern, and b) because other sources of N in prairie soils are limited, that grass or forb species will also become enriched in ^{15}N because of N transfer by mycorrhizae or through rapid uptake

from soil. N taken up by the plant can also be partitioned differently to roots, shoots and stems, so it is important to use the entire above-ground portion of the plant in measuring isotope discrimination, again an issue with deep-rooted species.A similar result can be achieved by direct fertilization of soil with ^{15}N containing fertilizer, with subsequent measurement of ^{14}N and ^{15}N in legume and control plants (Giller, 2001). An issue here is the high cost of ^{15}N and the variation in plant species across the prairie landscape.

3.6. Most probable number counts of rhizobia in soil

Rhizobia cannot easily be quantitatively recovered from soil and counted. Instead a common procedure is to prepare dilutions of the soil, and inoculate these into replicate tubes containing surface-sterilized and pregerminated seeds of an appropriate legume host. Following growth for several weeks in the growth chamber, the number of tubes at each soil dilution in which the host is nodulated is determined, with the number of rhizobia in the soil then estimated using a statistical procedure. Methods are detailed by Somasegaran and Hoben (1994).

3.7. Nodule occupancy and success in nodulation

Soils will often contain some indigenous rhizobia (Gaur and Lowther, 1980; Brockwell et al., 1982; Ellis et al 1984) that are infective for a newly introduced legume. Especially where native legumes are present, these indigenous rhizobia can be responsible for a significant proportion of the nodules formed (Brockwell et al., 1982; Ellis et al., 1984), and reduce the benefits from inoculation to the host under test (Weaver and Frederick, 1974a,b; Gaur and Lowther, 1980; Thies et al., 1991). Further, and while some inoculant strains may still dominate in the soil five to fifteen years after inoculation (Diatloff, 1977; Brunel et al., 1988; Lindstrom et al., 1990), not all inoculant strains are equally persistent in the soil (Chatel et al., 1968; Brockwell et al., 1982; Gibson et al., 1996). For many, their contribution to overall nodule formation in the years after inoculation can decline dramatically. Tests for the determination of inoculant strain(s) nodule occupancy on their hosts and their persistence are considered below. It should be noted that in the case of most crop plants these tests can be performed using nodules collected in the field, whereas with deep-rooted perennial legumes, this is difficult to do For such species rhizobia may need to be trapped from soil, collecting soil samples and using this as inoculum for inoculation of surface-sterilized seed of the appropriate legume or legumes (Tlusty et al., 2004). There is every possibility that this introduces a bias in the diversity of the rhizobia recovered, due to minor differences in compatibility of the selected host with the various rhizobia present in the soil (Laguerre et al., 2003; Slattery et al., 2004).

a) Serological determination of strain nodule occupancy: Essentially the only method used in inoculant-recovery and strain-diversity measurements from the 1940s to the 1980s (Vincent 1941, 1942; Ellis et al., 1984), this method uses differences in the antigenic composition of different species and strains of rhizobia to categorize rhizobia and identify specific inoculant or soil strains. Methods are described by Somasegaran and Hoben, (1994). Such procedures are labor intensive, requiring in many cases that antisera be prepared for each of the different inoculant strains in question; that inoculant strains used are serologically distinguishable from those indigenous to the soil, and from each other; and often that antisera be absorbed with other marginally reactive organisms to heighten their specificity before they are used in strain identification (Somasegaran and Hoben, 1994). Serological techniques have now given way largely to PCR-based methods of inoculant strain recognition.

b) Intrinsic antibiotic resistance patterns identify organisms on the basis of their tolerance to low

levels (1 - 20 ppm) of a range of different antibiotics (Josey et al., 1979). Difficulties are that this approach requires considerable set up work to establish resistance patterns for the inoculant strains, and of the soil rhizobia, and also is media intensive.
c) Antibiotic- or genetically tagged inoculant organisms identify organisms on the basis of their natural or induced resistance to high levels of antibiotic or to genetic markers such as the glucuronidase, alkaline phosphatase or luciferase genes (Bushby, 1981; Sessitsch et al., 1998). Methodology is relatively simple but can be complicated by antibiotic-resistance induced change in nodulation or nitrogen fixation traits, significantly weakening the inoculant organism. In many such studies this has not been adequately investigated. Natural resistance to antibiotics, for example the greater tolerance of Gram negative bacteria to bacitracin, can sometimes be used in the development of selective media (Louvrier et al., 1995).
d) Genetic fingerprinting uses a DNA primer and repetitive elements in the DNA of the organisms being studied to generate unique DNA bands, then amplifies these using the polymerase chain reaction (Versalovic et al., 1994; Rademaker and de Bruijn, 1997). Bands are then resolved by electrophoresis on agarose gels and viewed by staining with safe green or (less-desirably) ethydium bromide. Banding patterns are then used to generate similarity values for the different strains tested, with dendrograms then generated using clustering protocols such as possible with Bionumerics (Houston, TX). Examples of this approach applied to *Rhizobium* are given in recent papers from the *Rhizobium* Research Laboratory (Tlusty et al., 2005; Beyhaut et al, 2006b: Martir et al., 2007): detailed methodologies are given in Chapter 5. The procedure is moderately expensive to set up and run, and requires training to use, but allows high sample throughput.

3.8. Soil quality traits enhanced by inoculation and nitrogen fixation

Legume based cropping and ley systems have been the basis for sustainable production since the time of the Romans (Wild, 1988), in large measure because of their contribution to the organic carbon and nitrogen status of soils (Drinkwater, 1998, Dijkstra et al., 2006; Lemke et al., 2007). Beiderbeck et al (2005) noted that inclusion of legumes in a formerly fallow-wheat system enhanced microbial numbers in soil, increased microbial C and N 170% and 191% respectively, promoted C mineralization, and significantly enhanced phosphatase and aryl sulfatase activity, with significant increase in the relationship between microbial C and N and soil organic C and N. Microbiological attributes were more sensitive and responsive to the effects of legume residues than had been reported in previous studies. Similar results were observed in our previous MNDOT prairie project, even though the plots had been cut and raked to limit N availability (Graham et al., 2004), in CRP land after both short and long term enrollment (Baer et al., 2000) and in high diversity plots containing legumes (Djikstra et al., 2006).

3.9. Point in time assay of nitrogen (N_2) fixation

The enzyme nitrogenase is a non specific reductase having the ability to reduce a number of compounds including acetylene. When nodulated legumes are incubated in an atmosphere containing acetylene, this is reduced to ethylene, a compound easily quantified using gas chromatography (Hardy et al., 1968, 1973). A theoretical conversion factor between ethylene produced and nitrogen fixed has been suggested for the determination of nitrogen fixation rates, but can vary widely with environmental conditions. Weaver and Danso (1994) review this methodology and some of its problems, perhaps the greatest of which is that many investigators

are prone to extrapolate from a short incubation period to long-term rates of N_2 fixation. For prairie plants the acetylene reduction assay can be an effective tool for comparing short-term rates of fixation between treatments measured at the same growth stage, or with repeated assays made over the course of a growth season. However, given the depth of rooting system in such plants, the studies would have to be done in pots, and so have limited applicability to the field situation.

Determinate root nodules export the nitrogen fixed in symbiosis in the form of allantoin or allantoic acid, with sap concentration of these compounds strongly correlated to short-term rates of N_2 fixation (Herridge and Peoples, 1990). Unfortunately, of the major mid-western prairie legumes only *Desmodium* species have determinate nodules; with the remainder likely to transport the products of N_2 fixation in some other form.

3.10. Methodological constraints

Our inability to isolate rhizobia directly from soil limits strain collection, and biases recovery and diversity estimates where host-plant trapping is employed toward organisms with which the host is most compatible (Bernal and Graham, 2001; Mutch and Young, 2004). Further it is likely that the soil may contain more non-infective rhizobia than those that are infective (Segovia et al., 1991) while genetic rearrangement in the soil has rarely been quantified but could also contribute to differences between inoculant rhizobia and subsequent populations of soil organisms(Hungria et al., 2006; Nandasena et al., 2006, 2007). Inoculant establishment and microbial diversity estimates need to balance estimates based on host plant trapping with others obtained using rhizobia derived directly from soil. Unfortunately, direct recovery of rhizobia from soil using selective or semi-selective media remains problematic and difficult. A number of selective media have been published and are cited by Tong and Sadowsky (1994) and Louvrier et al. (1995), but rhizobial recovery direct from soil is still problematic and a constraint in studies such as this.

CHAPTER 4: Wheat and other grasses as carriers and beneficiaries of rhizobial and other inoculant microorganisms used in prairie establishment and function. Laboratory and field experiments

4.1. Background

Legume seed quantities used in prairie planting are small, with the numbers of rhizobia that can be applied using conventional seed inoculation low. Together with the fact that prairies are mainly planted in the Fall, with legume and rhizobia forced to survive harsh winter conditions and rhizobia to persist in soils until legumes establish, effective nodulation of the seeded legumes can be limited (Graham, 2005b). Greater inoculant numbers and more uniform distribution of the rhizobia can be achieved using granular, soil applied inoculants (Herridge, 2008) but perhaps because of cost, the inoculant strains for prairie legumes have rarely been supplied in this format. Another alternative, the inoculation of cereal or other plants grown as cover crops, with the subsequent transfer of the rhizobia to their appropriate legume host, has been available for many years (Diatloff, 1969) but in most agricultural situations makes little sense. One situation where its use does seem appropriate is with prairie plantings. Rhizobia are capable rhizosphere and endophytic organisms; already well known for their stimulatory, and primarily plant hormonal effects on the growth of cereals such as corn, rice and wheat (Hilali et al., 2001; Gutierrez Zamora et al., 2001; Rosenblueth and Martinez-Romero, 2004). For the latter, Doan and Graham (unpublished data, 2004) showed striking differences among wheat varieties in *Rhizobium* numbers supported in the rhizosphere, with older varieties such as Roughrider and Seward better able to support these organisms. We report here research on the inoculation of winter wheat and native grass species with rhizobia from prairie legumes, and on rhizobial persistence in the rhizosphere of this host, including both field and glasshouse studies. Our goal was to determine the feasibility of applying inoculant rhizobia through cereal rather than legume inoculation. We also wanted to determine whether winter wheat growth *per se* could be benefited by rhizobia or other plant growth-promoting bacteria.

4.2. Methods

Field evaluation of cereals and native grass species as alternate carriers of rhizobia, Becker 2004-2005, and 2007:

A field experiment planted at the Becker Sand Plain Experiment Station, Becker, MN in Fall, 2004 evaluated 33 winter wheat, rye, Canada wild rye, slender wheat grass, and oat cultivars (see Table 4.1) for persistence and growth in the field, and as inoculant carriers and surrogate hosts for the rhizobia associated with prairie legumes. Soil at the station is a Hubbard Loamy sand (sandy mixed frigid Entic Hapludoll [Zvomuya et al., 2003]); planting followed rye in 2003, and the soil was ploughed and packed before seeding. The site used was in the dryland area of the experiment station and received neither irrigation nor fertilization.

A randomized complete block design with four replications was used; each replicate containing a single 6m row of each cultivar sown at 120 seeds m^{-1}. Seeding of all but the oat varieties took place on September 17, 2004, with all seed preinoculated prior to planting with the mix of rhizobia for MNDOT prairie legumes recommended by Tlusty et al (2004). Sterile peat inoculant formulations for each legume species were used, and mixed immediately prior to application, with 40% gum arabic used as sticker. Inoculation was intended to supply 10^3 to 10^4 initial rhizobia per seed, and rows of inoculated grass- or cereal species were alternated with rows of inoculant-free commercial wheat to minimize cross contamination. This approach had

proved effective in earlier studies with Illinois bundle flower (*Desmanthus illinoensis*, Beyhaut et al. 2006a*).* Seeding of the five oat varieties occurred in early spring, 2005, and used the same inoculant formulations and practices as for the other plant species.

The cross-row technique of Howieson and Ewing (1986) was used to assess the degree of establishment of rhizobia in soil over winter and early spring, and to determine if rhizobia had spread from the initial site of inoculant placement in the seed row. For this, surface-sterilized seeds of the legumes *A. canadensis*, *D. purpurea*, and *D. canadense* were prepared (Somasegaran and Hoben, 1994) then planted as two-foot rows, parallel to the rows of cereal, and at distances of 5, 25, and 40 cm. from these rows. Initial planting of the cross rows was undertaken on June 5th, 2005, with the legume rows then carefully dug out on July 22, 2005 and assessed for the percent nodulated plants of each legume, and distance from the initial rows of inoculated seed. Legume plants recovered in the field were stored over ice pending return to the laboratory, then inoculant-strain nodule occupancy determined using BOXA1R-PCR (Tlusty et al., 2005, see detailed methodology in Chapter 5).

Growth of the cereals and gramineous hosts for *Rhizobium* was evaluated on two occasions in 2005, first for persistence and growth at the time of cross row planting, then on July 30th, 2005 for plant development and tillering (Feekes, 1941). These parameters are still those recommended by R. Klein (Univ. of Nebraska, pers. comm. 2008) as most appropriate for the measurement of cereal growth and benefit from rhizosphere bacteria.

A second field experiment contrasting the older wheat cultivar Roughrider with the more recent introduction Oklee was planted at Becker in summer 2007. This sought to contrast these cultivars in their ability to maintain rhizobial viability under field conditions, and was also intended to evaluate potential interactions among *Rhizobium* strains in a mixed inoculant. The field used had no history of legumes, and had been cropped to cereals in previous seasons. Treatments included the two cultivars, and contrasted seed inoculation with sterile peat culture of strain UMR6808 with a treatment in which strains UMR6355, UMR6437 and UMR 6808 were applied as a mixed inoculant.The experimental design was a randomized split-block with four replicates (see Figure 4.1), and the experiment was carried out without N fertilization, as in normal prairie planting. Twenty five days after planting, one foot segments of row were carefully dug-up and taken in a cooler to the laboratory. Serial (1:4) dilutions of whole root washings were used to inoculate *Dalea purpurea* plants with *Rhizobium* numbers on the root then determined using the most probable number procedure of Somasegaran and Hoben (1994). Poor wheat growth under the low fertility conditions and poor rainfall in the 2007 summer precluded yield harvest of the wheat.

Wheat cultivar- *Rhizobium* studies under growth chamber and glasshouse conditions:

Initial experiments to further examine wheat cultivar/*Rhizobium* strain interaction under growthchamber and glasshouse conditions, and especially to explore strain specificities and interactions, used magenta units prepared and sterilized as detailed by Tlusty et al (2004) and surface-sterilized wheat seeds pregerminated prior to planting, then inoculated with known numbers of *Rhizobium* suspended in sterile dilution fluid. These experiments were discontinued when control treatments that had been inoculated with rhizobia, but contained no wheat plant, achieved similar cell numbers to those with inoculated wheat plants. Inoculated sterile soil has been used in the preservation of bacteria (Jensen, 1961), but the growth levels achieved in this study in the absence of the cereal precluded any evaluation of wheat/rhizobial strain interaction.

Table 4.1. Cereals tested as alternate carriers of rhizobia, Becker, 2004.

Winter wheat	Rye	Canada wild rye	Oat	Slender wheatgrass
ReGreen	Wintergrazer	Canada wild rye [A]	Andrew	Unknown
Crimson	Rymin	Canada wild rye [B]	Moore	
Jerry	Homil 21	Canada wild rye [C]	Ogle	
Ransom	Wrens 96	Canada wild rye [D]	Gopher	
Roughrider	Oklon		Clinland 64	
Seward				
Roughrider				
Expedition				
Wahoo				
Harding				
Elkhorn				
Agassiz				
McClintock				
Wesley				
CDC Buteo				
Jagelene				
Millenium				
Wendy				

[A] CWR from Prairie Restor. Inc.

[B] CWR from Prairie Moon Nursery

[C] CWR from Ecological Prairie Systems

[D] CWR from Shooting Star Native Seeds

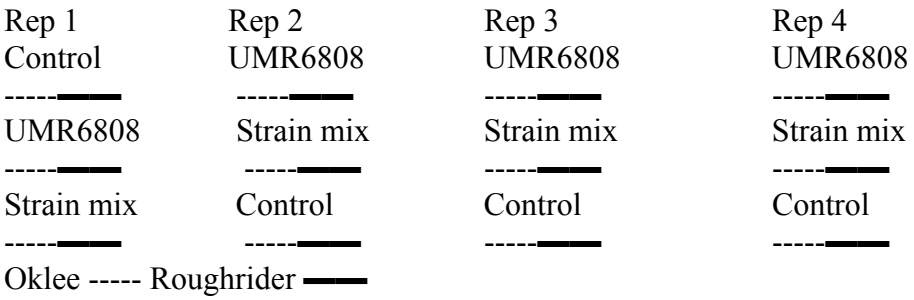


Figure 4.1. Becker wheat-rhizobia field experimental design, 2007.

Instead, pot studies were initiated which were open to the environment, but attempted to control contamination through sterile watering and minimal disturbance of the soil surface. Because of the difficulty of counting rhizobia under the less-than-aseptic conditions of the glasshouse environment, these studies were paralleled by attempts to develop a semi-selective medium for fast-growing rhizobia. Martinez et al. (1991) and Amarger et al. (1997) earlier noted the tolerance of *R.etli, R.gallicum* and *R.giardinii* (all isolated from *Phaseolus vulgaris*) to nalidixic acid. Based on their experiences, we first evaluated fast-growing *Rhizobium* strains from *Dalea, Desmanthus, Astragalus, Amorpha* and *Robinia* for tolerance to 50 ug mL^{-1} nalidixic acid, then combined elements of the synthetic medium of Parker and Oakley (cited by Graham, 1963) and the semi-selective medium of Louvrier et al (1995) to formulate a medium that could be used in the direct isolation of fast-growing rhizobia from soil. This medium (POGL2) contained lactose and NH_4NO_3 as energy and N sources respectively, the fungicides cycloheximide, pentachloronitrobenzene and congo red, and nalidixic acid).

For the pot studies of wheat/rhizobial-strain interaction, seeds of Roughrider winter wheat were surface-sterilized using 75% EtOH and 3% hypochlorite followed by 5 rinses in sterile distilled water (Somasegaran and Hoben, 1994) then pregerminated for 3 days in sterile sand trays. Several different inoculant treatments were compared in this study:

a) single strain inoculations using UMR6808, 6437 or 6355 as the inoculant strain
b) multiple strain treatments in which all three strains were added together.
c) interaction of single and multiple rhizobial strain inoculants with plant growth-promoting (PCPR) A*zospirillum* (EMD Biosciences, Milwaukee, WI) or *Bacillus* (Becker-Underwood, Ames, IA).
d) control treatments without wheat, in which the wheat was not inoculated, or in which legume seeds were inoculated and planted normally.

Inoculant rhizobia were grown in AG liquid medium with aeration (Somasegaran and Hoben, 1994), with cells counted using a Petroff-Hausser counter (Hausser Scientific, Horsham, PA) and their concentration adjusted to 10^8 cells mL^{-1} with dilution fluid (Gherna, 1994). Separately surface-sterilized and pregerminated wheat seedlings were then soaked in dilute inoculant culture or an equal volume mixture of all three strains, for 30 min. before seeding. Inoculation of the PGPR organisms was as recommended by the manufacturer. Twelve seeds were planted per 6" pot into initially sterile sand culture, but with nutrients and water supplied as half-strength Summerfield nutrient solution with 10 ppm N (Summerfield et al., 1977) through capped watering tubes. Pots were open to the atmosphere and free-draining.

Initial harvests were made 14 days after seeding with 6 plants pot^{-1} harvested and evaluated for shoot length and biomass and one plant per treatment tested for microbial numbers in the wheat rhizosphere. Roots and adhering soil from this plant were used to prepare a dilution series, with counts of rhizobia then made using the Miles-Mizra plating method and three different plating media**;** the non-selective BYMA+ congo red, the synthetic POGL2 medium for selective growth of fast-growing rhizobia, and BJSM medium (Tong and Sadowsky, 1994). BJSM was designed for selective isolation of soybean rhizobia from soil, but also supported the growth of all *Bradyrhizobium* spp prairie inoculant strains we tested. Counts from the wheat rhizosphere were made after only a short growth period to minimize opportunities for contamination under glasshouse conditions, and because of the number of treatments and platings involved, used only three of the four replicates. Pure cultures of UMR6808 and UMR6437 were always included as controls to ensure that counts from the rhizosphere dilution series were made at times appropriate to the organisms they contained and to the plating medium

used. When realistic counts of rhizobia-like colonies were obtained on POGL plates inoculated with dilutions prepared from plants inoculated withUMR6808, the organisms growing on POGL medium were validated as rhizobia by picking a single colony from each of the 24 plate counts, suspending these in dilution fluid and using this as inoculant applied to surface-sterilized seedlings of *Dalea purpurea* grown in magenta units. Extent of nodulation was then determined after 7 weeks growth in the glasshouse.

Following this initial evaluation the pots together with the wheat plants they contained were subject to a 3-week period of cold, with temperatures in the cold room shuttled between 2^{o}C and -4^{o}C, to "simulate" winter conditions, and to further stress rhizobia in the wheat rhizosphere. Pots were then returned to the glasshouse, and each planted to one-week old surface-sterilized seedlings of *Dalea purpurea, Desmodium canadense* and *Astragalus canadensis.* Conditions and watering regime were as for wheat, with the legumes harvested after 6 weeks and evaluated for growth, nodule number and nodule mass plant^{-1}.

Differential promotion of wheat growth by rhizobial inoculants:

Potential benefit to winter wheat root growth from rhizobial inoculation was also assessed. Three-day old broth cultures of UMR6355, UMR1899 and a five-day old broth of the slower-growing UMR6437 were tested for their ability to promote early root growth in three wheat varieties Oklee, Roughrider and Harding. Evenly sized seeds were imbibed overnight in broth cultures filtered to remove rhizobia or with untreated broth cultures, then ten seeds per strain treatment placed on the surface of individual Petri dishes containing 1.0% water-agar. Plates were incubated at 28º C for 72 h in the dark, then maximum root length determined. We had previously compared several assay durations, and concluded that three days was optimal.

4.3. Results

Field evaluation of cereals and native grass species as alternate carriers of rhizobia, Becker 2004-2005, and 2007:

With the exception of Wrens 96 rye and regreen winter wheat all fall-planted cereals and grasses tested regrew the following season, though marked differences in vigor were noted. Five groups could be distinguished at the July 30th evaluation, with the oats still in vegetative stage at this time, and the Canada wild rye lines just beginning to flower. Eleven of the winter wheats and one rye line were at the kernels watery ripe stage (Feekes 10.5.4), while a further seven winter wheat and three rye lines were at the mealy ripe stage of development (Feekes 11.2). Rye varieties had less tillers ft of row^{-1} than the winter wheat lines (an average of 29 and 36.2 respectively) with highest tiller number ft^{-1} of row found among the winter wheat cultivars Expedition, Seward, Agassiz, Crimson, and Roughrider, all with more than 40 tillers ft^{-1} row. Plant phenological differences are summarized in Table 4.2.

Table 4.2. Cereal lines classified according to phenologic stage and mean number of tillers.

	Species	Variety	Phenologic stage	Mean of tillers ft-1
Group 1	Oat	Oat 5 Andrew	Tillers formed (3)	n/a
Oat	Oat	Oat 4 Moore	Tillers formed (3)	n/a
	Oat	Oat 1 Ogle	Tillers formed (3)	n/a
	Oat	Oat 3 Gopher	Tillers formed (3)	n/a
	Oat	Oat 2 Clinland 64	Tillers formed (3)	n/a
Group 2	Canada wild rye	CWRye A	Beginning flowering (10.5.1)	n/a
Canada wild rye	Canada wild rye	CWRye B	Beginning flowering (10.5.1)	n/a
	Canada wild rye	CWRye C	Beginning flowering (10.5.1)	n/a
	Canada wild rye	CWRye D	Beginning flowering (10.5.1)	n/a
	Slender w. grass	Slender w. grass	Beginning flowering (10.5.1)	n/a
Group 3	Wheat	Seward	Kernels watery ripe (10.5.4)	48.00
wheat	Wheat	Agassiz	Kernels watery ripe (10.5.4)	44.00
	Wheat	Roughrider	Kernels watery ripe (10.5.4)	41.25
	Wheat	Jerry	Kernels watery ripe (10.5.4)	36.50
	Wheat	Mc Clintock	Kernels watery ripe (10.5.4)	36.50
	Wheat	Wahoo	Kernels watery ripe (10.5.4)	33.25
	Wheat	Millenium	Kernels watery ripe (10.5.4)	30.50
	Wheat	Harding	Kernels watery ripe (10.5.4)	24.00
	Wheat	CDC Buteo	Kernels watery ripe (10.5.4)	22.25
Group 4	Wheat	Expedition	Mealy ripe (11.2)	48.25
wheat	Wheat	Crimson	Mealy ripe (11.2)	43.25
	Wheat	Elkhorn	Mealy ripe (11.2)	39.75
	Wheat	Ransom	Mealy ripe (11.2)	39.75
	Wheat	Wesley	Mealy ripe (11.2)	39.5
	Wheat	Wendy	Mealy ripe (11.2)	33.5
	Wheat	Jagelene	Mealy ripe (11.2)	23
Group 5	Rye	Wintergrazer	Mealy ripe (11.2)	20.5
rye	Rye	Homil 21	Mealy ripe (11.2)	40
	Rye	Oklon	Mealy ripe (11.2)	34
	Rye	Rymin	Kernels watery ripe (10.5.4)	38.25

Numbers between parenthesis are Feekes phenologic stages

1=emergence, 2=beginnning of tillering, 3=tillers formed, 4=beginning of the erect growth, 5=leaf sheaths strongly erected, 6=first node visible, 7=second node of stem formed, 8=flag leaf visible, 9=ligule of flag leaf visible, 10=boot stage, 11=ripening.

When rhizobial survival over-winter was assessed July 22, 2005 using *A. canadensis*, *D. purpurea*, and *D. canadense* as trap species, the percent nodulation across all grass and cereal species tested was greatest with *Desmodium canadense* and least for *Astragalus canadensis*. With *Desmodium* and *Dalea* there was little variation in % of nodulated trap host plants according to the cereal cultivar inoculated and planted in 2004. However with *Astragalus canadensis,* legume seeds planted adjacent to the variety Agassiz achieved substantially greater % nodulation, while those associated with the wild ryes showing no nodulation (Figure 4.2).

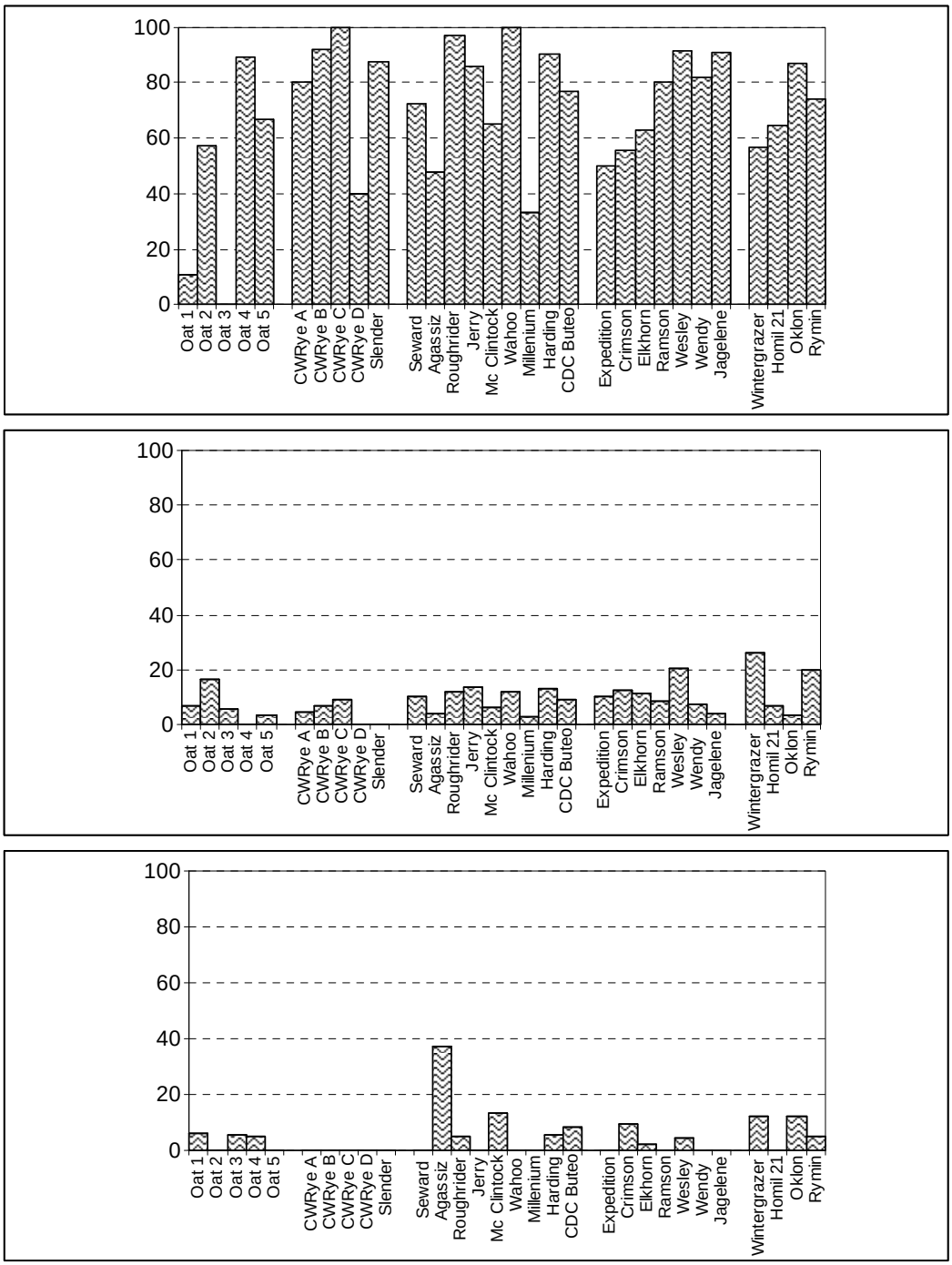


Figure 4.2. Percent nodulated plants for each trapping host averaged across cross-row distance. From top to bottom: *Desmodium canadense*, *Dalea purpurea*, and A*stragalus canadensis.*

Effect of distance from the initial point of inoculation on % nodulated *Desmodium* and *Dalea* plants for five promising winter wheat lines (Crimson, Harding, Roughrider, Wahoo, and Wesley) are shown in Figure 4.3. Data for *Astragalus* is not included in this figure because of the low percentage of *Astragalus* plants bearing nodules.

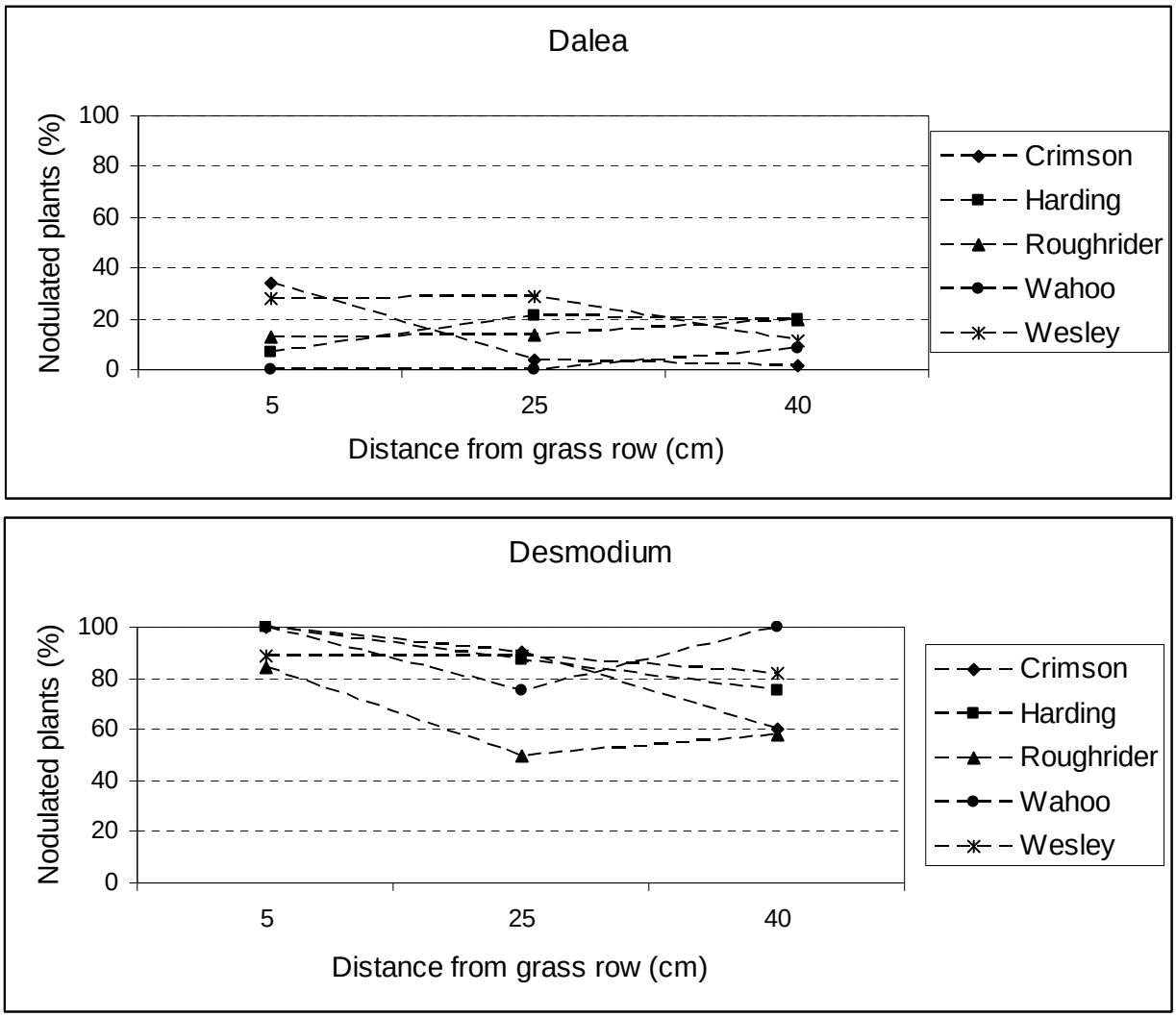


Figure 4.3. Percent of nodulated plants over distance from previous year grass row, for *D. purpurea* and *D. canadense*. Becker, 2004-5.

Given poor rainfall in 2005, only a limited number of nodules and rhizobia could be recovered for determination of strain nodule occupancy. For isolates from *Desmodium* evidence of similarity to the slow-growing inoculant strains used was not as strong as we would have liked, though 28 of 34 *Desmodium* rhizobia recovered did cluster into a broad group that included the inoculant strain UMR6437. Results were better with the rhizobial strains recovered from *Dalea*, with more than 50% of the strains from this host clustering with the inoculant strain UMR6808. Interestingly, almost 25% of the nodules recovered from *Astragalus* contained the inoculant strain UMR7520 for *Amorpha.* Both *Amorpha and Astragalus* rhizobia belong in the genus *Mesorhizobium,* and this is not the first time that host-swapping for these two species has been reported (Tlusty et al., 2005, see also Graham et al., 2004).

When the rhizobial populations associated with field-grown wheat plants of the cultivars Roughrider and Oklee were determined using an MPN count with *D. purpurea* as host, the counts were greater for rhizobia from roots of Roughrider than for Oklee, irrespective of whether

single- or mixed-strain inoculants were used. Counts of *Dalea* nodulating rhizobia per root averaged 3 x10^5 and 8 x 10^5 vs. 2.1 x10^3 and 5.3 x 10^4 for Roughrider single inoculation, Roughrider mixed inoculation, Oklee single inoculation, and Oklee mixed inoculation, respectively.

Development of a semi-selective medium for the growth of *Rhizobiu*:

Growth of diverse strains of rhizobia known to occur in Minnesota prairie soils or adjacent agricultural fields following subculture on POGL2 medium is shown in Table 4.3. POGL2 clearly supports the growth of the majority of fast-growing *Rhizobium* strains indigenous to the Minnesota prairie, while *Mesorhizobium, Sinorhizobium* and *Bradyrhizobium* strains grow poorly, if at all, on this medium. Equally interesting from a biogeographic perspective is that the medium does not appear to support the growth of *Rhizobium* strains of European origin, for example *R.leguminosarum* isolates from peas and clovers. To further test this medium, plate counts were made on POGL2 medium using Becker prairie soil samples that had been in cold storage for 6 months. Plates were not totally without contamination, but with most it was possible to make approximate counts, and to make numerous isolations from colonies with the characteristics of fast-growing rhizobia. It appears from these results that this medium has significant potential as a semiselective medium for fast-growing *Rhizobium,* and warrants additional study. With the collaboration of Dr. M.A. Graham (USDA-ARS Soybean Genomics Laboratory at Iowa State University) we have identified PCR-primers for a 660 base region of the *gyrA*-gene that is involved in nalidixic acid tolerance (Boonmar et al., 2007). With these primers both *R.etli* and *R.leguminosarum* produce a single band of similar size following PCR. The consensus sequence for this section of the genome of *R.etli* (+ ve) and *R.leguminosarum* (- ve) shows a number of single nucleotide differences between these species; with restriction enzyme/sequencing studies now underway to identify those regions needed for resistance, and to develop species specific probes for use in further biodiversity studies. These studies will continue beyond the present project, and emphasize direct recovery of *Rhizobium* from prairie soil, and their comparison with strains isolated by host-plant trapping.

Inoculation of winter wheat with single- and mixed rhizobial inoculant strains, *Azospirillum* and *Bacillus*:

Effect of inoculation with single and multiple strains of rhizobia, and of *Azosprillum* and *Bacillus* on the germination and early growth of Roughrider winter wheat are shown in Table 4.4. Inoculation with UMR6355 + *Azospirillum* had a marked positive effect on wheat germination and early plant growth, whereas inoculation with *Bacillus* or several *Bacillus/Rhizobium* combinations tended to inhibit germination and early growth. Counts of rhizobia on wheat roots measured 14 days after germination varied in their success. We had anticipated problems in counts made on BYMA + congo red because it is a broadly utilized growth medium with little control of contaminant organisms. Prevention of contamination of our pots would have been needed to effect counts using this medium, and this was a difficult goal. Concern with the use of this medium was one of the reasons we included a determination of nodulation success after freezing and thawing treatments later in the experiment. We were surprised by the poor results with the selective BJSM medium. We had previously shown good growth of inoculant *Bradyrhizobium* strains on this medium, without major contamination problems. In the event bacterial rather than fungal contamination was worse than anticipated, and precluded effective counts in any of the pots where bradyrhizobia were used as inoculant. In

Table 4.3. Growth of different groups of rhizobia on POGL2 medium containing 50 μg per mL sodium nalidixate.

Species	Strains tested	Isolated colonies in <10 days YES	NO	% positive
Rhizobium				
R.etli	15	13	2	87
R.etli-like	12	10	2	83
R.gallicum	21	21	-	100
R.giardini	11	11	-	100
R.tropici	1	1	-	100
R.leguminosarum	10	0	10	0
R.galegae	1	0	1	0
Mesorhizobium spp. (*huakuii, amorphae, tianshanense, loti*)				
	20	(2)	18	10
Sinorhizobium spp (*meliloti, medicae, fredii, ciceri)*				
	12	3	9	25
Bradyrhizobium spp (japonicum, elkanii, canariense, spp)				
	11	0	11	0
Rhizobium species not known with certainty. Isolates are from the hosts indicated				
Coronilla varia	1	1		
Onobrychis viciifolia	2	2		
Oxytropis	1	1		
Psoralea spp.	4	2		
Robinia psuedoacacia	3	3		

() borderline result

contrast results with *Dalea* rhizobia based on serial dilution on POGL medium were excellent (see Table 4.5) with *Rhizobium* counts from the wheat rhizosphere commonly greater than 10^7 cells per root, and significantly greater than obtained in the uninoculated controls. While no control plant showed nodulation, 12 of the 24 "*Rhizobium"* colonies picked from plates used to count rhizobia, and tested for nodule formation on *Dalea purpurea* nodulated this host, a percentage only marginally less than normally achieved in magenta units. At the very least, use of POGL medium should permit quantitative direct recovery of *Rhizobium* strains from soil, and further studies are planned to evaluate counts and diversity of rhizobia recovered directly from a range of prairie systems. While BJSM medium was not effective in the isolation of *Bradyrhizobium* from soil in the present study, we have subsequently shown that inclusion of bacitracin (40 ug mL^{-1}) in this medium improves selectivity (data not included). The opportunity to contrast host-plant-trapped and directly isolated slow- and fast-growing rhizobia from the same soil, and how these may be differentially affected by seed source and environmental conditions, justifies persistence and further studies with both media.

Figure 4.4 shows differences in *Desmodium* nodulation and plant prowth when pots containing wheat plants used as a surrogate host for single or multiple strains of rhizobia, *Bacillus* or *Azospirillum*, and cold stressed, were then planted with surface-sterilized seedlings of *Desmodium canadense* and grown for several weeks under glasshouse conditions. As in most inoculation studies there was a clear correlation between subsequent nodule development and

Desmodium plant growth ($r^2 = 0.6430$), with two treatments outperforming the remainder. These were the positive control in which pregerminated *Desmodium* seedlings were inoculated with UMR6437 and planted without stress as the other treatments were being removed from the cold chamber; and the treatment in which wheat was inoculated with UMR6437, with the pots then planted to *Desmodium* after the cold temperature treatment. Treatments in which multiple strains of rhizobium, or rhizobia plus *Bacillus* or *Azospirillum* were applied together did not do as well, though better than the treatment in which inoculant was soil applied without the wheat host.

Table 4.4. Effect of inoculation with single and multiple strains of *Rhizobium,* and of *Azospirillum* and *Bacillus* on the germination and early growth of Roughrider winter wheat.

Inoculation Treatment	% Germination[1]	Shoot length[2] (cm)	Shoot dry weight[2] (g)
UMR6808 alone	70	22.7	0.158
UMR6808 + *Azospirillum*	66	21.7	0.162
UMR6808 +*Bacillus*	66	22.0	0.163
UMR6808, 6437 and 6355	60	20.7	0.137
Mixed rhizobia + *Azospirillum*	68	22.2	0.154
Mixed rhizobia + *Bacillus*	50	20.3	0.126
UMR6437 alone	68	19.5	0.136
UMR6437 + *Azospirillum*	66	22.5	0.151
UMR6437 + *Bacillus*	62	19.0	0.123
UMR6355 alone	66	22.5	0.151
UMR6355 + *Azospirillum*	85	23.2	0.150
UMR6355 + *Bacillus*	75	23.0	0.169
Azospirillum alone	72	20.2	0.142
Bacillus alone	56	19.0	0.134
No Inoculation	70	22.2	0.147

[1] four replications each of 12 plants, measured 7 days after seeding
[2] four replications each of 6 plants measured 14 days after planting

Table 4.5. Counts of *Rhizobium gallicum* UMR 6808 obtained by serial dilution and direct count on selective POGL medium from the root systems of 14 day-old wheat plants. Treatmetns included inoculation with UMR6808 alone, or with multiple inoculant rhizobia and plant growth promoting bacteria.

Inoculant treatment	*Rhizobium* count (log_{10} $plant^{-1}$)			
	Rep 1	Rep 2	Rep 3	Average
UMR6808	7.0969	7.7501	7.8750	7.6804
6808 + *Azospirillum*	7.2430	7.1383	7.0000	7.0142
6808 + *Bacillus*	6.9420	7.3979	6.7403	7.0650
UMR6808 + 6355+ 6437	7.6409	7.3979	6.7403	7.4586
3 rhizobia + *Azospirillum*	7.5118	7.3273	7.3010	7.3906
3 rhizobia + *Bacillus*	7.9098	7.8372	7.4948	7.7811
Uninoculated control	0	0	5.9452	5.4771

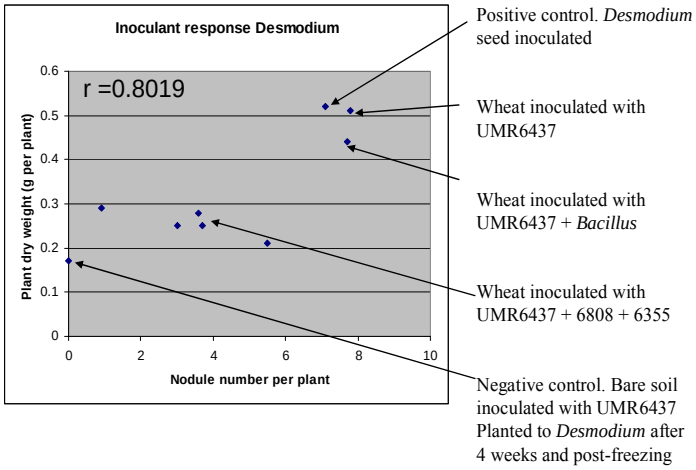


Figure 4.4. Effect of single- or multiple-strain inoculation of wheat on the nodulation and growth of surface sterilized *Desmodium canadense* seeded after the wheat and following a period of simulated winter.

Dalea and *Astragalus* seedlings planted into the dry surface conditions of our pots exhibited very high mortality, and though we sometimes found as many as 4 nodules per plant, the number of surviving plants and the variation in nodulation and plant growth limited meaningful consideration of the results.

Differential promotion of wheat growth by rhizobial inoculants:

Mean maximum root length of wheat plants grown on water agar plates for three days in the presence of filtered or unfiltered culture exudate are shown in Figure 4.5. With Roughrider all treatments resulted in root elongation, with the filtered preparation from UMR6437 most active, Treatments with filtered UMR6437 increased average maximum root length for both Roughrider and Harding, 50% and 35% respectively, a finding that could have significant agronomic application. Host/strain interactions were also evident, with the inhibition of root elongation of "Oklee" by strain UMR6355 most striking, and differences between the filtered and unfiltered UMR1899 preparations with all three cultivars notable. O'Connell and Handelsman (1993) reported *R.tropici* to produce a toxin inducing leaf chlorosis in beans; no negative responses with other plant species have been noted.

4.4. Conclusions

In this series of field and glasshouse trials we have used a number of wheat cultivars as surrogate hosts for *Rhizobium*. Cultivar differences have been noted, with lines such as Crimson, Harding, Roughrider, Wahoo and Wesley showing both plant vigor and reasonable rhizobial recovery. We have seen nothing clearly better than Roughrider, and regard it as the wheat cultivar of choice for further studies. We plan to evaluate the rhizosphere processes contributing to its support of *Rhizobium,* and whether or not rhizobia inoculated onto this host are capable of acting as endophytes.

In this chapter we have reported studies in which we compared single and multiple strain inoculants (the latter contained rhizobia needed for different legumes and/or biocontrol organisms), with variable results. Thus for *Dalea* rhizobia tested on both Oklee and Roughrider there appeared to be little effect of multiple strain inoculation on early rhizosphere numbers, though effects of *Bacillus* and *Azospirillum* addition were noted. The effect did not appear to be as great when mixed inoculants plus PGPR were used. With *Desmodium,* use of inoculants containing strains specific for other species of legumes did impact *Desmodium* nodulation and plant growth, but we would need additional studies to determine whether this was simply due to overcrowding of the wheat rhizosphere or to antagonistic interaction between strains.

In the studies reported here we had some difficulty in recovering inoculant strains using host-plant trapping, and in showing the spread of rhizobia using the parallel row methodologies of Howieson and Ewing (1986). For these techniques to have been uniformly successful we would have needed irrigation of our prairie areas and surface watering of pot cultures. The former could have resulted in artificially enhanced plant growth, soil microbial populations and rhizobial movement; the latter would favor contamination and unnatural spread of the inoculant strains. In the field studies the % nodulation of *Desmodium* as a trap host was much greater than for *Dalea,* while inoculant strain recovery was more clear cut with *Dalea* than with *Desmodium.* The latter genus is recognized for promiscuity and ability to nodulate with diverse rhizobia… but is often ineffective in such symbioses (Date, 1991). Thus numerous indigenous or non-inoculant rhizobia could have contributed to nodulation, and have lowered inoculant strain recovery. It is interesting that we did not have this very high level of nodulation by apparently indigenous rhizobia in the full prairie studies reported in Chapter 5.

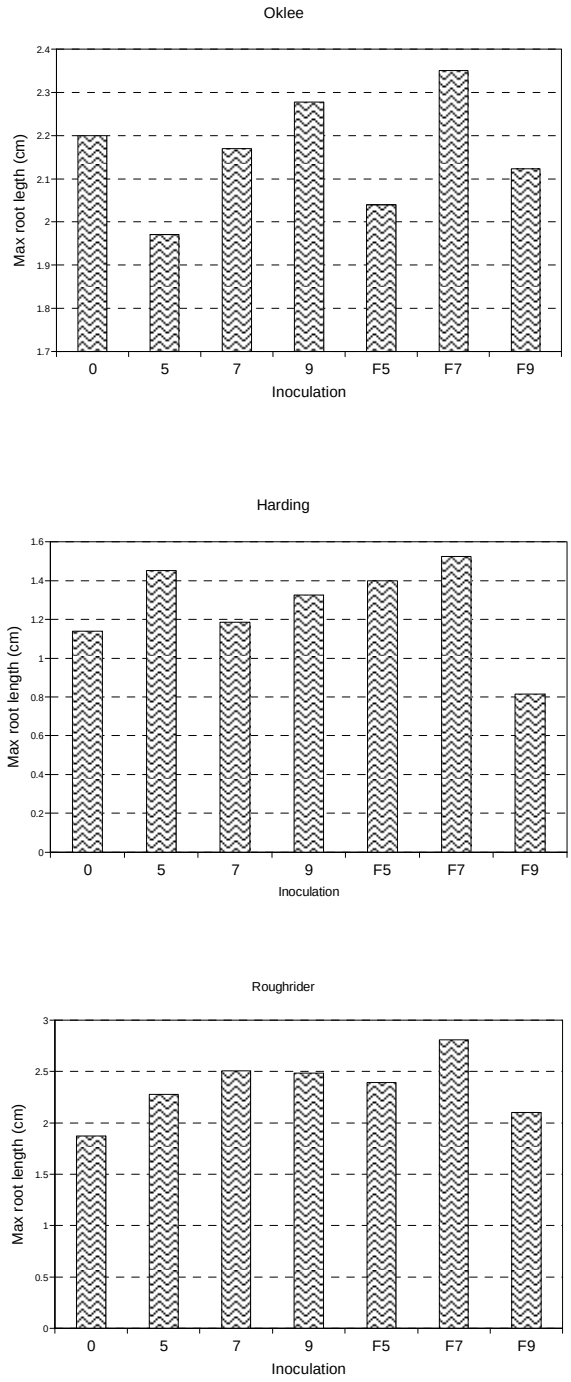


Figure 4.5. Mean maximum root length of Oklee, Hardee and Roughrider wheats on 1% water agar after three days incubation at 28 ºC. Treatments: 0=uninoculated broth, 5=UMR6355, 7=UMR6437, 9=UMR1899, F5=filtered UMR6355, F7=filtered UMR6437, F9=filtered UMR1899. Values are means of 10 replicates.

To count and isolate *Rhizobium* directly from soil we developed a semi-selective medium. In turn this led to a primer for PCR based on part of the *gyr*-gene, that has potential as a species specific probe for *Rhizobium.* More studies are needed to validate the use of POGL as a semiselective medium for the direct isolation of *Rhizobium* from soil. However, even if it is only moderately selective, the ability to obtain significant numbers of rhizobia directly from soil and to be able to compare host-plant trapped and directly isolated strains, will be a powerful tool in studying differences in *Rhizobium* diversity and biases introduced by host plant trapping. *gyr*-based species specific probes for fast-growing rhizobia will also have value in high-throughput strain recognition.

CHAPTER 5: Inoculation method, and the establishment and contribution of legumes and rhizobia to the developing prairie. Becker Field studies 2004-2008

5.1. Background

Natural ecosystems are commonly limited in nitrogen (Seastedt and Knapp, 1993) and after fire or grazing, can be dependent on leguminous plants for nitrogen inputs (Towne and Knapp, 1996). Because of this, prairie seed mixtures used by the Minnesota Department of Transportation in roadside revegetation and wetland reconstruction activities include a number of indigenous legumes, among them *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea purpurea* and *D.candida, Desmodium canadense* and *Lespedeza capitata.* Difficulties in the successful inoculation of these legumes is discussed in Chapter 2, see also Graham (2005).

In a previous MNDOT study (Graham et al., 2004) we identified new inoculant cultures for the legumes being emphasized by MNDOT, and undertook prairie establishment studies at the Becker Sandplain Experiment station. It was clear from these studies that supply of adequate numbers of inoculant rhizobia was often an issue. This chapter reports studies to examine alternate methods of inoculation that could help to overcome this constraint. We also monitor the development, nodulation, and nitrogen fixation of the legume species included in the seed mix, and their contribution to improved soil quality and plant species composition in the prairie ecosystem

5.2. Methods

Prairie establishment:

The prairie area used in this study was established in the dryland area of the University of Minnesota Sandplain Research Station at Becker, MN (45º24' N, 93º53' W) in Fall 2004, and covers an area of 110 x 43 m. Soil at the station is a Hubbard Loamy sand (sandy, mixed frigid Entic Hapludoll [Zvomuya et al., 2003]). The area had been in a cereal-fallow rotation for a number of years before this planting, and was in fallow during the summer and early fall of 2004. The experimental design was a randomized complete block with five treatments and four replicates, with each plot measuring 16.8 X 7.6 m, and the plots separated by 3 m wide buffer strips as shown in Fig. 5.1. The inoculation treatments used in this study were:

- granular clay-based inoculant (Becker Underwood, Saskatoon,Ca)
- granular peat-based inoculant (Becker Underwood, Saskatoon, Ca)
- seed applied powdered peat inoculant
- inoculated wheat seed applied as a cover crop
- uninoculated (control).

The same rhizobia were used in the preparation of each inoculant (see Table 5.1). Each was grown separately in BYMB liquid medium (Graham, 1963), cell numbers then determined using a Petroff-Hausser counter (Horsham, Pa), and the strains mixed to provide equal numbers of each in the inoculant. Inoculant for the legume- and wheat-seed inoculation treatments were applied the night before seeding, using PBX (Rizobacter, Argentina) sticker and the proportions of sticker/inoculant and seed recommended by Somasegaran and Hoben (1994). Granular inoculants were soil applied at 10 kg ha^{-1}, and raked in during seeding.

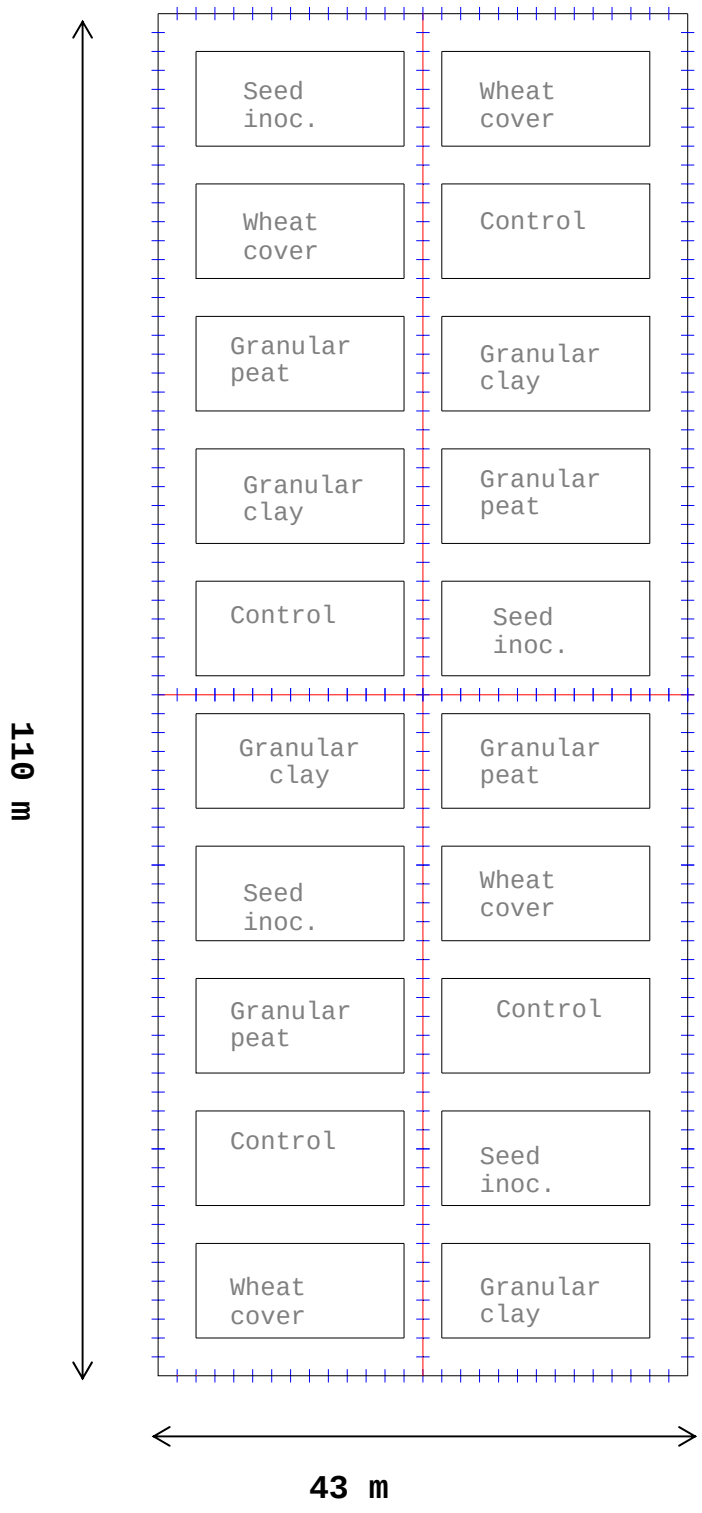


Figure 5.1. Becker prairie plot layout showing the distribution of inoculation treatments and replications.

Table 5.1. Rhizobial species and strains used in the 2004 Becker prairie seeding.

Plant species	Microsymbiont	UMR Strain No(s)
Amorpha canescens	*Mesorhizobium amorphae*	7520
Astragalus canadensis	*M.huakuii*	6355
Chamaecrista fasciculata	*Bradyrhizobium spp*	6404, 6437
Dalea candida	*Rhizobium spp.*	6808, 7205, 7240
Dalea purpurea	*Rhizobium spp.*	6808, 7205, 7240
Desmodium canadense	*Bradyrhizobium spp*	6617, 6437
Lespedeza capitata	*Bradyrhizobium spp.*	6513, 6564

Soil was ploughed and packed prior to seeding. Inoculated seed of Roughrider winter wheat was machine planted in the appropriate plots in mid-September. Soil in the remaining plots was ploughed and packed a second time after the first hard frost, with seeding of the prairie grasses, forbs and legumes then undertaken on November 18th, 2004. Seed used in prairie establishment was provided by Prairie Moon Nursery (Winona, MN), and corresponded to their GM-025 seed mix as shown in Table 5.2. It was applied at a seeding rate of 750 seeds m^{-2}. For seeding, grasses and non-legume forb species were broadcast using a Vicon fertilizer spreader and raked in. Subsequently, 1.5-m wide strips were marked across the experimental area and inoculated- or uninoculated-legume seed and granular inoculants carefully applied using glass bottles with perforated metal caps ("cheese shakers") as applicators. Finally, plots and buffer strips were raked, with care taken to avoid transfer of inoculant between plots. Field workers wore sterile paper boots to limit cross-contamination, with the boots changed and equipment disinfected with 90% ethanol after each plot.

Plant community establishment and legume contribution to the Becker prairie, 2005-2008:

Plant community development has been monitored each July since establishment, using the semi-quantitative Relevé method (Mueller-Dombois and Ellenberg, 1974) applied to the entire plot as a sampling unit, and recording cover/abundance and sociability for each species, and estimates of bare soil. In addition, 1.4 m^2 permanent quadrats, embedded in the plots in September 2005, have been used to monitor plant abundance and productivity, with the plant material recovered from each quadrat, separated by guild, then dried and plant biomass determined for each. In July, 2008 sub-samples taken from these quadrats were dried and ground then subject to total nitrogen analysis using the Dumas method (Simone et al., 1994; Matejovic, 1995).

Soil chemical and physical analysis:

Soil samples for determination of chemical and biological properties have been taken each year since establishment with five 15 cm x 2 cm cores collected per plot, and combined to obtain one composite sample per plot for both soil and microbiological analysis. Routine chemical and physical analysis was undertaken at the University of Minnesota Research Analytical Laboratory using standard procedures detailed on their website (http://ral.cfans.umn.edu/). Samples taken for analysis in 2007 were also analyzed for microbial

Table 5.2. Composition of the seed mix used in the 2004 Becker prairie seeding.

Scientific name	Common name	Seeds/ha
Forbs		
Agastache foeniculum	Anise Hyssop	161,463
Asclepias tuberosa	Butterly Weed	69,428
Asclepis verticilata	Whorled Milkweed	39,469
Aster azureus	Sky Blue Aster	430,549
Ceanothus americanus	New Jersey Tea	40,901
Coreopsis patmata	Prairie Coreopsis	53,819
Echinacea angustifolia	Narrow-leaved Coneflower	125,580
Eryngeum yuccifolium	Rattlesnake Master	121,093
Gentiana quinquefolia	Stiff Gentian	387,518
Helianthus occidentalis	Western Sunflower	50,232
Liatris aspera	Button Blazing Star	172,224
Monarda fistulosa	Wild Bergamot	251,164
Monarda punctata	Spotted Bee Balm	322,925
Penstemon grandiflorus	Large-flowered Beardtongue	100,463
Ratibida pinnata	Yellow Coneflower	107,642
Rudbeckia hirta	Black-eyed Susan	297,086
Tradescantia ohiensis	Ohio Spiderwort	57,407
Verbena stricta	Hoary Vervain	100,465
Zizia aurea	Golden Alexanders	59,200
Legumes		
Astragalus canadensis	Canadian Milk Vetch	243,075
Baptisia leucantha	White Wild Indigo	18,299
Chamaecrista fasciculata	Partridge Pea	193,746
Desmodium canadense	Showy Tick Trefoil	29,600
Lespedeza capitata	Round-headed Bush Clover	86,985
Dalea candida	White Prairie Clover	204,516
Dalea purpurea	Purple Prairie Clover	193,750
Dalea villosa	Silky Prairie Clover	150,695
Amorpha canescens	Lead Plant	172,224
Amorpha nana	Fragrant False Indigo	107,640
C4 Grasses		
Andropogon scoparius	Little Bluestem	1,614,573
Bouteloua curtipendula	Side-oats Grama	452,085
C3 Grasses		
Elymus canadensis	Canada Wild Rye	279,859
Koeleria cristata	June Grass	1,076,374

biomass carbon, biomass nitrogen and respiration (Jenkinson and Powlson [1976] as described by Parkinson and Paul [1982]) in the laboratory of Dr Deborah Allan.These additional soil

parameters were chosen as representative of those commonly used in the evaluation of short-term changes in soil quality with treatment.

***Rhizobium* recovery and inoculant strain contribution**:

In each field season (2005 to 2007) representative, composite soil cores were collected from the prairie plots in July, then used in trapping rhizobia, with *Astragalus canadensis*, *Dalea purpurea*, and *Desmodium candense* used as trap hosts. The exact procedure has varied with year, but initially used sterile magenta units (Tlusty et al., 2004) planted with surface-sterilized pregerminated seedlings of each host. Magenta units were inoculated with 5 mL of a 10^{-1} dilution from one of the plot samples and grown in the growth chamber for 10 weeks at 25ºC/20ºC day/night temperature, 14 hour photoperiod, and 90% relative humidity. At harvest, presence or absence of nodules and nodule number per plant were recorded, then the nodules collected and preserved at -80^{o}C until analyzed by PCR. This procedure was modified in 2007 because rhizobial isolates obtained using magenta units in the first two years had not been sufficient for detailed analysis of inoculant strain success, and because we believed that this was critical in the third year of testing. Accordingly, ten 500 mL capacity polystyrene coffee cups were used per host and treatment, and small drainage holes drilled at their base. Cups were filled with sterile soil, watered with sterile plant nutrient solution, and inoculated as for the magenta units, then capped with a lid through which four 1 cm holes were burned. Three seedlings were then planted per cup, with the fourth hole used for a large capacity drinking straw through which sterile plant nutrient solution could be added every two days. When *Desmodium* and *Astragalus* seedlings did not grow well in this system, large 75 x 25 cm covered trays were used, and several hundred seedlings grown and inoculated per sample plot.

***Rhizobium* characterization using the polymerase chain reaction (PCR):**

Rhizobia derived from nodules by host plant trapping were characterized using repetitive sequence-based polymerase chain reaction (RSB–PCR) with BOXA1R as primer (Versalovic et al. 1994). Nodules were thawed and allowed to imbibe in sterile distilled water, then surface sterilized by soaking for 1 minute in 95% ethyl alcohol, followed by rinsing in sterile distilled water, and immersion in 60% Chlorox solution for 1 minute (Vincent, 1970). They were then rinsed repeatedly in sterile distilled water. Surface-sterilized nodules were then crushed and streaked onto plates of yeast-mannitol agar medium (Vincent, 1970), incubated 6-10 days at 28°C, then isolated colonies selected and re-subcultured. Single colonies picked from each isolate were stored on slants at 5°C and separately in 20% sucrose/10% peptone solution at -70^{o}C. Standard reference strains and the inoculant cultures used in prairie inoculation were grown on the same medium and under the same conditions. At several points in this study we felt it necessary to confirm the identity of particular isolates as *Rhizobium*. For this, isolates in question were evaluated for nodulation and nitrogen fixation with their recommended host using the magenta system as described by Tlusty et al. (2004).

In preparation for PCR analysis rhizobia from a single colony on a 4-10 day old YMA plate (depending on rhizobial species) were used to charge a 1 µl sterile disposable inoculating loop, and this inoculum added to 1 mL sterile tryptone-yeast extract broth medium (TY, Somasegaran and Hoben, 1994) in a microfuge tube. Following growth with agitation at 280 rpm for 24 h at 24^{o}C, cell suspensions were centrifuged at 16,000 g for 2 minutes, the supernatant discarded, and the cells washed in 1 mL sterile 1 M NaCl. The cell preparations were then re-centrifuged for 4 minutes at 16,000 g, the supernatant discarded, and the pellets resuspended in

0.5 mL sterile TRIS-EDTA (TE 10:1, pH 7.6) buffer (Sambrook et al., 1989) and stored at -20°C until used. For PCR, each cell preparation was further diluted 1 in 4 in sterile double-distilled water, just before use, with 20 µL quantities then transferred to sterile 96-well trays and kept at -20°C pending PCR. PCR was carried out using C-09 BOXA1R primer (Versalovic et al. 1994, Integrated DNA Technologies, Coralville, IA), a PTC 200 thermocycler (MJ Research, Waltham, MA), and the procedure of Rademaker and de Bruijn (1997). Each set of reactions included a negative control. Ten µL subsamples of PCR product were then separated by electrophoresis using 20 x 25 cm horizontal gels containing 1.5% agarose, with the gels run at a constant temperature of 8^{o}C at 70V for 17.5 h. Three 1kb DNA ladders (Promega Corp. Madison, WI) and the PCR product of the strains *Rhizobium spp* UMR6815 and *Rhizobium etli* UMR1632 were included for reference in each gel. Following electrophoresis, gels were stained in 0.5 µg/ml ethidium bromide or SYBR Green 1 for 30 minutes, then photographed using a FOTO/Analyst Archiver (Fotodyne Inc., Hartland, WI). Gel images were subject to qualitative analysis using Bionumerics version 3.5^{+} (Applied Maths, Sint-Martens-Latem, Belgium) with band positions on each gel normalized by comparison to bands (200 to 2000 bp) in the 1Kb ladder. The densitometric curve for each strain was analyzed using Pearson's correlation coefficient as a measure of similarity, with clustering analysis based on the unweighted pair group method with arithmetic averages (UPGMA) then used to generate dendrograms showing the degree of similarity among isolates and reference strains. In some studies a multidimensional scaling (MDS) plot (BioNumerics version 3.5^{+} was used as a visual representation of all the rhizobia recovered and analyzed.

5.3. Results

Soil characteristics:

Substantial plot to plot variation in results of soil chemical analysis made it difficult to determine any specific soil treatment effects. This was exacerbated by year to year effects. Thus slight increase in pH in essentially all plots between the 2005 and 2006 analyses (see Figure 5.2), were offset by a decline in soil pH in 16 of the 20 plots between 2006 and 2007. Soil properties for the different prairie plots three years after establishment are shown in Table 5.3, with each data point averaged for four repetitions and multiple samples per plot. The high Ca level in the inoculated wheat treatment was due to a single soil sample that tested extremely high for Ca; the other replicate samples were not significantly different from values obtained with the other treatments. We do not have an explanation for this aberration; no other differences were obvious.

Changes in soil organic matter, soil % carbon and nitrogen and microbial biomass as a consequence of inoculation treatment are shown in Table 5.4. In this study, and in the earlier prairie studies at Becker (Graham et al., 2004) we raked off leaf litter, and used periodic fire as a means to lower soil N and to drive the prairie toward greater dependance on legumes. It is notable in the present study that even though % soil organic matter, % C and % N declined in three of the five inoculation treatments over time, there was substantial increase in microbial biomass N in the granular clay, granular peat and inoculated wheat treatments. Microbial biomass N is commonly used to measure short term soil quality changes. Improvements in microbial biomass C and N were in general less than reported in the earlier 2004 study, perhaps because of the difficult growing conditions of 2006 and 2007. Differences in microbial biomass

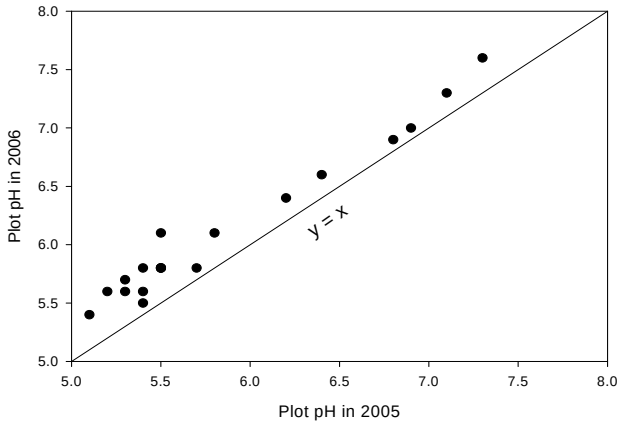


Figure 5.2. Change in soil pH in the 20 prairie plots between 2005 (the year of establishment) and 2006. Each dot corresponds to one of the five inoculation treatments and four repetitions of the Becker study.

Table 5.3. Soil chemical analysis of the Becker prairie Plots, 2007[1,2].

Inoculation treatment	pH	Bray P	K	Ca	Mg
		←		ppm	→
Uninoculated	6.0	53	112	442	62
Seed inoculated	5.8	39	80	497	61
Granular clay inoculated	5.6	45	91	397	54
Granular peat inoculated	5.7	38	100	445	58
Inoculated wheat	6.3	45	85	767	52

[1] Methods of analysis are described on the Analytical services website at http:ral.coafes.umn.edu/soil.htm

[2] Each value represents multiple samples per plot and is the mean of four repetitions

C and N between soil from the 1999 prairie, the various treatments of the present study, and from adjacent agricultural fields, make it evident that continuous agriculture draws down soil microbial C and N, and that original levels are not rapidly restored.

Prairie establishment and development:

Weeds from the soil seed bank predominated, and the percentage of bare soil was relatively high, in the growing season after establishment (Table 5.5). Annual weeds predominated with *Lepidium virginicum* (Virginia pepperweed), *Crepis tectorum* L. (Narrow-leaved hawk's beard), *Ambrosia artemisiifolia*(Ragweed), *Berteroa incana* (Hoary alyssum), *Chenopodium album* L. (Common lamb's quarters), *Conyza canadensis* (Marestail/Horseweed) and *Setaria pumila* (Yellow foxtail) conspicuous. The perennials

Convolvulus arvensis (Field bindweed) and *Silene latifolia* ssp. alba (White cockle) also covered significant areas of some plots. Isolated prairie plants occurring in most plots included *Penstemon grandiflorus* (Large-flowered Beardtongue), *Monarda punctata* (Spotted Bee Balm),

Table 5.4. Changes in soil organic matter composition, and microbial biomass C and N three years after prairie establishment [1], and comparisons with both an agricultural field and an older prairie restoration[2].

Inoculation treatment	Soil Trait[1]						
	% OM	% C	%N	C/N ratio	<----------Microbial----------→ Respiration[3]	Biomass C[3]	Biomass N[3]
Not inoculated	1.9	1.23	0.11	15.5	4.17	88.53	7.65
Seed Inoculated	1.6	0.89	0.07	14.6	2.32	84.64	6.04
Gran. Clay Inoculated	1.4	0.79	0.05	15.9	2.19	103.92	9.57
Gran. Peat Inoculated	1.5	0.93	0.05	17.9	1.84	98.88	8.01
Inoculated Wheat	2.0	1.40	0.09	17.4	3.23	110.88	9.67
Agric. Field	ND	0.96	0.06	16.0	2.88	74.03	6.96
8 year old prairie	ND	1.18	0.07	18.0	5.60	173.19	18.27

[1] Each value is derived from composite samples per plot and is the average of four replicates
[2] Respectively a rye/fallow rotation and the 1999 prairie established in the dryland area of the Becker sandplain experiment station.
[3] Method of Jenkinson and Powlson [1976] as described by Parkinson and Paul [1982])

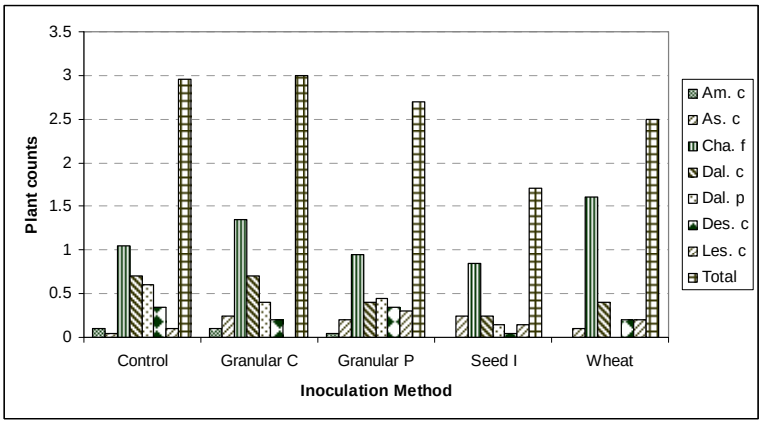
Table 5.5. Summary of plant community development, Relevé data, Becker 2005[1].

	Relevé Number[2]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Common name	Scientific name																				
Virginia pepperweed	*Lepidium virginicum*	3		2	2	2	2	2	3	2	3	2	2	3	3	2	1	2		2	1
Field bindweed	*Convolvulus arvensis*	2	4	4	4		3	2	1		2	1	2			2	4	1	4	2	
White cockle	*Silene latifolia* ssp. *alba*	5	1			1	2	2	1	3	3		2	2	3	2			2	2	1
N-leaved hawk's bear	*Crepis tectorum L.*			r	r					3		3	2	3	1						
Ragweed	*Ambrosia artemisiifolia*							1	r		r	r			r	r					
Hoary alyssum	*Berteroa incana*								3								1	2		1	
Common Lamb´s qua	*Chenopodium album* l	1	1			2		1	1				1		1	1	1				
Marestail/Horseweed	*Conyza canadensis*	1	3	2	2	1	2		1		r	r		r	r						
Yellow foxtail	*Setaria pumila*			r	r	r	r	r				r			r	r	r	r			
Bare soil		1	3	4	4	3		1	2	1	1		2	1	1	3	3	3	3	3	1

[1] Numbers are Braun-Blanquet cover-abundance classes:
r =single, 1=1-5%, 2=5-25%, 3=25-50%, 4=50-75%, 5=75-100%
[2] for simplicity and because no differences were found associated with inoculation treatments, Relevés are numbered based on spatial contiguity

Coreopsis palmata (Prairie coreopsis), and *Rudbekia hirta* (Black-eyed Susan). When legumes in the 1.4 m^2 quadrats were counted, *C. fasciculata* and *D. candida* (White prairie clover) were most common, while *A. canadensis* (Canadian Milk Vetch, seeded at 243,095 seeds ha^{-1}), *A.*

canescens (Lead plant, seeded at 172,224 seeds ha^{-1}), and *L. capitata* (Round-headed Bush Clover) were almost absent (Figure 5.3). The highest total count for any legume was for *C. fasciculata* with 1.6 plants per quadrat in the 'wheat cover' inoculation treatment.



[1] Values are means of 20 observations (4 replicates x 5 1.4m^2 quadrats)

Figure 5.3. Counts of individual and total legumes in the different inoculation treatment of the Becker prairie, 2005[1].

Plant community development was evaluated again in mid July 2006, with the change in plant community composition very evident. Where weeds had dominated in the previous growing season, few weeds were now evident and the percent bare soil was significantly reduced. Instead, a small group of prairie plants including *Rudbekia hirta* (Black-eyed Susan), *Koeleria cristata* (June grass), *Monarda punctata* (Spotted Bee Balm), *Monarda fistulosa* (Wild Bergamot), *Elymus canadensis* (Canada Wild Rye), *Penstemon grandiflorus* (Large-flowered Beardtongue), and to a lesser degree *Andropogon scoparius* (Little Bluestem) were evident with abundance ratings from less than 1% to 25-50% (Table 5.6).

Legume numbers in the five 1.4 m^2 quadrats per plot evaluated in 2006, were almost double those found in 2005, with the most commonly found legumes somewhat different from those of the previous year. *D.candida* (White prairie clover), *D. purpurea* (Purple prairie clover) and *D. canadense* (Showy tick trefoil) were predominant, with *C. fasciculata* (Partridge pea) accounting for a smaller proportion of the total legumes than in the previous year, and *L. capitata* (Round-headed bush clover) and *As.canadensis* (Canadian milk vetch) still only rarely encountered, and *Am. canescens* (Lead plant) only common in the granular clay treatment (Figure 5.4). There was a clear effect of inoculation treatment, with total legumes in the granular clay treatment more that double those in the control plots, and legumes in the wheat cover treatment also increased. Drought conditions prevailed for much of the second half of the growing season, with rainfall to August 28th, 2006 4.55 inches below average. Plants were extremely stressed for the rest of the growing season, and no further evaluations were attempted.

Plant biomass data for the different inoculation treatments in 2007 is shown in Table 5.7, with legume biomass least in the seed inoculated treatment, and greatest in those treatments with higher rates of inoculation. 2007 was again a drought year at Becker, though some precipitation fell late in the growing season. Because of these conditions we postponed collection of data on total nitrogen per plot and per plant guild per plot as affected by inoculation treatment until

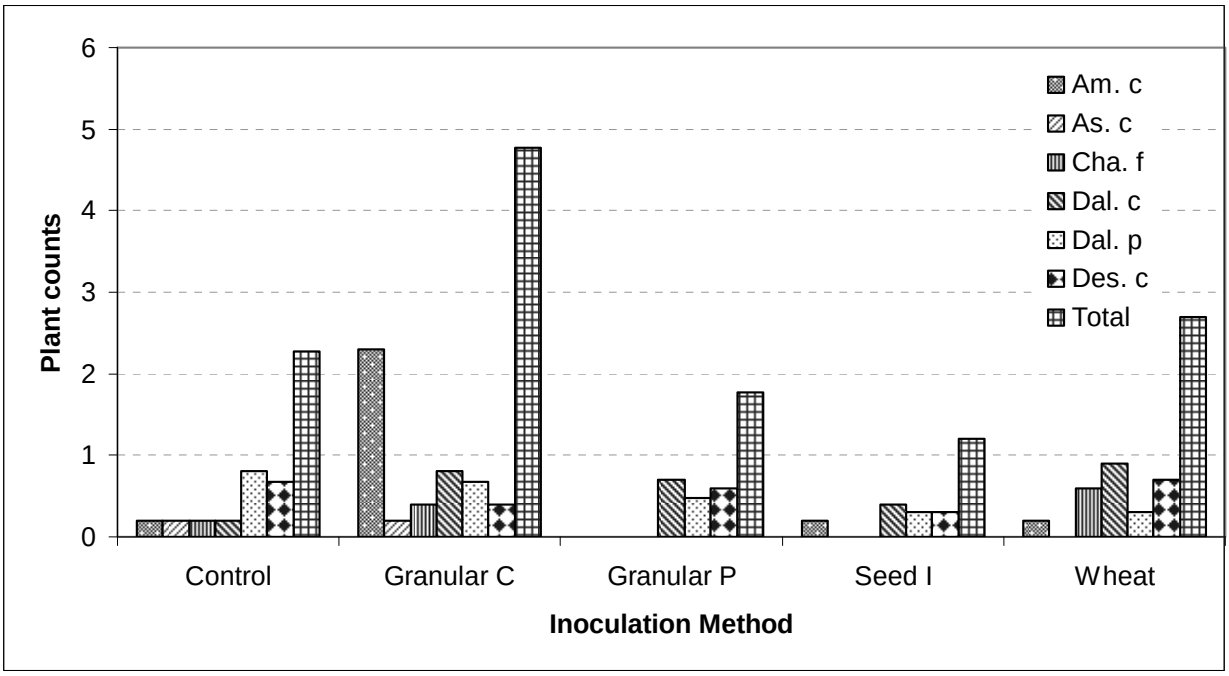
Table 5.6. Summary of plant community development, Relevé data, Becker 2006[1].

Treatment	Rep	Species						
		Rud.h	Koe.c	Mon.p	Mon.f	Ely.c	Pen.g	And.s
Control	1	2	2	2	+	1	1	
	2	2	2	2	+		1	
	3	2	1	2	1	2	1	
	4	2	+	2	2	2		
Gran C	1	2	1	2		+	1	
	2	2	2	2	+	1	1	
	3		2	2	1	1	1	
	4	3	1	2	1	1	1	
Gran P	1	2	1	2		+	1	
	2	2	2	2		1	1	
	3	2	2	2		1	1	
	4	2	2	2	1	1	1	
Seed I	1	2	2	2	1		1	
	2	3	1	1	2	2	1	
	3	2	2	2	1	1	1	
	4	3	1	2	2	1	1	
Wheat	1	2	1	2	+	1	1	2
	2	1	2	2		1	3	
	3	2	1	2		1		
	4	1	1	2	+	1		

[1] Numbers are Braun-Blanquet cover-abundance classes: + =<1%, 1=1-5%, 2=5-25%, 3=25-50%. Rud.h=*Rudbekia hirta* (Black-eyed Susan); Koe.c=*Koeleria cristata* (June grass); Mon.p=*Monarda punctata* (Spotted Bee Balm); Mon.f=*Monarda fistulosa* (Wild Bergamot); Ely.c=*Elymus canadensis* (Canada Wild Rye); Pen.g=*Penstemon grandiflorus* (Large-flowered Beardtongue), And.s=*Andropogon scoparius* (Little Bluestem).

spring 2008. Variation in number of legumes counted on a per plot basis in 2007 is shown in Table 5.8., with the total number of legumes per plot, and the numbers for each of the three major species *Lespedeza capitata, Dalea purpurea* and *D. candida*, greatest in the granular-clay based inoculant treatment. *Desmodium, Astragalus, Chamaecrista* and *Baptisia* establishment was poor, with only 63, 61, 45, and 16 plants, respectively, in the entire prairie area. Over the years of experimentation at Becker change in the performance of *Lespedeza capitata* has been very noticeable. In the initial years these plants suffered badly from grey leaf mould, and did not flourish. More recently (and perhaps as a result of warmer spring and fall periods, and drier to droughty conditions they have come to be important in the prairie mix.

Data collected in 2008 for grass, forb and legume plant dry matter production (g m^{-2}) and total N (g m^{-2}) in the plots with different inoculation treatments is shown in Table 5.9a and 5.9b. While variation between the plots was still substantial, notable in this data is the increased production and total N content of the granular peat and clay treatments, and the higher % N and total N of grasses in the granular clay and inoculated wheat treatments. This will warrant re-evaluation using larger plot areas, and legume species comparisons as the prairies age.



[1] Values are means of 20 observations (4 replicates X 5 1.4 m^2 quadrats)

Figure 5.4. Counts of individual and total legumes in the different inoculation treatments of the Becker prairie, 2006[1].

Table 5.7. Grass, Forb and Legume Biomass (g/m^2) in prairie plots receiving different inoculation treatments. Becker 2007. Data is the average of 5 1.4 m^2 quadrants per plot and 4replicate plots.

Inoculation Treatment	Grasses	Forbs	Legumes	Total
Not inoculated	242.8	64.6	3.02	280.42
Seed inoculated	186.7	64.2	1.21	252.11
Peat granular inoculant	176.2	60.7	4.39	241.29
Clay granular inoculated	155.2	53.2	5.15	213.55
Inoculated wheat	237.2	26.7	4.06	267.96

Inoculant strain recovery from the Becker prairie:

Because of the depth of their rooting system, we have never attempted to collect nodules directly from plants in the field. Rather, we have taken several soil cores from each plot, combined them to obtain a single soil sample for each plot, then used Magenta assemblies (Tlusty et al 2004) with *Dalea purpurea, Desmodium canadense* and *Astragalus canadensis* as trap hosts to recover rhizobia from the soil. The legumes used in prairie restoration are commonly promiscuous in the rhizobia with which they will associate (Bushnell and Sarles, 1937; Wilson, 1944; Tlusty et al., 2005), with the hosts we use nodulated predominantly by

Table 5.8. Frequency of the principal legumes (*Lespedeza capitata, Dalea purpurea* and *D. candida*) in prairie plots receiving different inoculation treatments, Becker, 2007[1].

	Lespedeza capitata	*Dalea purpurea*	*Dalea candida*	Total Legumes
Uninoculated	36.2	39	14.8	90.0
Seed inoculated	23.5	28.5	16	68.0
Granular clay inoculated	50.5	50.5	35.3	133.3
Granular peat inoculated	46	31.3	19	96.3
Wheat inoculated	35.5	45.5	16.8	97.8

[1] Counts in 2007 were done on a whole plot basis

Table 5.9. Influence of inoculation treatment on biomass and N recovery from grasses, forbs and legumes in the Becker prairies as a consequence of inoculation treatment. 2008.

a) Dry matter production

	Dry matter production ← g m^{-2}			Total biomass →
	Grasses	Forbs	Legumes	
Not inoculated	318.0	60.4	3.8	382.2
Seed Inoculated	276.1	56.5	0.9	333.5
Granular peat inoculated	239.8	64.6	7.4	311.8
Granular clay inoculated	284.5	46.2	10.6	341.3
Inoculated wheat seed	305.0	28.6	2.47	336.1

b) Percent and Total nitrogen recovery

	%N			← Total N (g m^{-2})			→
	Grasses	Forbs	Legumes	Grasses	Forbs	Legumes	Biomass N
Not inoculated	1.14	1.21	2.13	3.62	0.73	0.08	4.43
Seed inoculated	1.16	1.21	2.77	3.20	0.68	0.02	3.90
Granular peat inoculated	1.16	1.29	2.13	2.78	0.83	0.16	3.77
Granular clay inoculated	1.33	1.27	2.53	3.78	0.59	0.27	4.64
Inoculated wheat seed	1.41	1.64	2.68	4.30	0.47	0.07	4.84

Rhizobium, Bradyrhizobium and *Mesorhizobium* species, respectively. Table 5.10 shows the percentage of nodulated plants and nodule number per plant for each of the three hosts used in 2005 and 2006 sampling. For all three trap species in 2005, and for *Dalea* and *Astragalus* in 2006, there was a marked difference in % nodulated plants and nodule number per plant between the control and inoculated plots. Number of nodules per individual plant also varied considerably, affecting estimates of diversity and % inoculant strain recovery. Results for *Dalea* in 2006 declined from those in 2005, perhaps as the result of low seasonal precipitation, but with uninoculated controls would suggest that not all were due to inoculation (see also PCR data for 2006). In this study results with *Desmodium* have been highly variable (see for example the

Table 5.10. Nodulation data for three legume species used as trap hosts for soil rhizobia in the Becker prairie in 2005 and 2006.

	Inoculation treatment				
	Control	Seed Inoc.	Granular Clay	Granular Peat	Wheat
a) 2005 growing season.					
Dalea purpurea					
% Nodulated plants	25	50	69	53	63
Nodule number	9	29	32	29	46
Nodules/ nodulated plant	2.3	3.6	2.9	3.6	3.8
Astragalus canadensis					
% Nodulated plants	47	62	60	68	13
Nodule number	16	43	25	31	3
Nodules/nodulated plant	2.0	4.3	2.1	1.5	1.5
Desmodium canadense					
% Nodulated plants	50	53	61	45	44
Nodule number	12	16	17	18	22
Nodule/nodulated plant	1.5	2.0	1.5	2.0	3.1
b) 2006 growing season					
Dalea purpurea					
% Nodulated plants	6	37	30	21	33
Nodule number	12	20	23	45	16
Nodules/ nodulated plant	12	3.3	7.5	5.8	3.2
Astragalus canadensis					
% Nodulated plants	42	62	37	65	47
Nodule number	10	21	10	27	17
Nodules/nodulated plant	1.7	2.1	1.7	2.5	1.9
Desmodium canadense					
% Nodulated plants	87	62	37	65	87
Nodule number	37	21	35	49	56
Nodules/nodulated plant	2.6	2.1	1.7	3.1	4.0

difference in % nodulated plants for 2006 and 2007. and the low incidence of nodules recovered in the seed inoculation treatment in 2007). Drought and the lower incidence of *Desmodium* plants in some plots could have led to spatial effects, influencing the rhizobia recovered.

Rhizobium and *Bradyrhizobium* recovery from soil in the Becker prairie areas (host plant trapping with *Dalea purpurea* and *Desmodium canadense*, respectively) in 2007 is shown in Table 5.11. A response to inoculation in *Dalea* is evident in % plants nodulated across treatments, with the granular clay and peat preparations, superior, and nodule number per plant also greater than in previous testings. Results from trapping with *Desmodium canadense* as host in 2007 were disappointing, with additional plants needed to provide nodules for PCR analysis. More than 1000 sterile seedlings were planted to recover only 323 nodules. There was also marked variation in the frequency of nodulation between treatments, with 62 of 68 nodules

recovered from the control treatment obtained from a single soil sample and repetition. We could not trap rhizobia from *Astragalus* in 2007 because of problems in seed germination. We planted

Table 5.11. *Rhizobium* and *Bradyrhizobium* recovery from soil in the Becker prairie areas (host plant trapping with *Dalea purpurea* and *Desmodium canadense*,respectively) in 2007, three years after prairie establishment and inoculation.

		Inoculation treatment			
	Control	Seed Inoc.	Granular Clay	Granular Peat	Wheat
a) Using *Dalea purpurea* as trap host.					
No. of seedlings planted	120	120	120	120	120
No. of plants with nodules	28	15	36	39	19
% plants nodulated	23.3	12.5	30	32.5	15.8
No of nodules recovered	123	90	161	155	76
Nodules/nodulated plant	4.4	6.0	4.5	4.0	4.0
b) Using *Desmodium canadense* as trap host					
No. seedlings planted	251	261	278	277	236
No. with nodules	19	2	59	20	33
% plants nodulated	7.6	0.8	21.2	7.2	14.0
No of nodules recovered	38	2	101	44	138
Nodules/nodulated plant	2.0	1.0	1.7	2.2	4.2

seed numbers similar to those for *Desmodium,* but recovered less than 10 nodules total. Stable *Astragalus* seedling populations and nodulation have been a constant issue over the period since 2000 in which we have been working on the Becker prairies. Because of this, and the long preparation time in some of our experiments needed for cold moist stratification of seed, we have also undertaken an experiment to test the effect of infusion of seed with germination- and growth promoting bacteria (see p 61).

Rhizobia trapped from soil in each growing season were subject to Box A1R-PCR, the number of strains evaluated varying from 200 and 175 in 2005 and 2006, respectively, to more than 500 in 2007. Because of the short time between inoculation and first sampling in 2005, we will emphasize only the 2006 and 2007 results. The 175 nodule isolates trapped from soil in the 2006 growing season clustered into five major groups at a similarity level close to 80% (Figure 5.5). In three of these the rhizobia corresponded mainly to isolates trapped from *Desmodium*. Unexpectedly, however, 20 of the strains clustering with these groups were isolates from *Astragalus.* We cannot explain the *Desmodium /Astragalus* result. Rhizobia from *Desmodium* are generally considered *Bradyrhizobium spp* while those from *Astragalus* strains are usually classified as *Mesorhizobium huakuii* (Graham, 2008a). Given how little we know of cross-inoculation among prairie legumes and their rhizobia, it is quite possible that some slow-growing rhizobia have the ability to nodulate *Astragalus;* the reverse situation with *Desmodium* nodulated by both fast- and slow-growing rhizobia is well documented Hurse and Date, 1992; Chen et al., 1997), with some fast-growing isolates from *Desmodium* recovered in the 2007 sampling.

Further studies in this area are needed but are outside the mandate for this project. The other two clusters of rhizobia identified in 2006 BOX A1R-PCR analysis contained only isolates from *D. purpurea,* with one of these also including two of the inoculant strains UMR 7205 and UMR6808. In this dendrogram only 25% of the rhizobia from *Dalea,* 4% of those from *Desmodium,* and no *Astragalus* rhizobia identified with the inoculant strains used.

Box AIR-PCR of 315 *Dalea* rhizobia from those isolated in 2007 identified eight different clusters of *Rhizobium,* with strains in four of these clusters associated at greater than 80% similarity with one or other of the *Dalea* strains used as inoculants (Table 5.12). Given this level of similarity it appears that a high percentage of the *Dalea* rhizobia recovered are the result of inoculation. Overall 55% of these strains behaved similarly in their PCR banding patterns to the *Dalea* inoculant rhizobia, but this varied considerably with inoculation treatment. Thus only 2% of the strains recovered from the seed inoculation treatment showed homology to any inoculant rhizobia, whereas 53%, 92% and 100% of the granular clay, granular peat and wheat cover-crop inoculated strains, respectively, appear to have been derived from an inoculant strain. The low values for % inoculant strain recovery in the seed inoculation and control treatments, and the much greater values and very satisfactory values obtained with the granular soil-applied and cover-crop inoculated treatments, show again the importance of *Rhizobium* application rate on inoculation success. We would anticipate spatial differences in the distribution of the rhizobia between treatments and plan further studies in this area. Based on simplicity alone our preference would be to emphasize rhizobial inoculation using a granular peat format.

Table 5.12. Distribution of *Rhizobium spp* (*Dalea*) strains from prairie plots at the Becker Sandplain Research Station according to initial inoculation treatment and PCR Cluster. [1] Indicates strains clustering with an inoculant strain.

PCR	Initial inoculation treatment, Fall, 2004					
Cluster	Not Inoculated	Seed Inoculated	Gran Clay. Inoculated	Gran Peat Inoculated	Wheat Inoculated	Total
I	0	0	5	8	24	37[1]
II	0	0	0	13	4	17[1]
III	24	1	44	23	2	94[1]
IV	0	0	0	24	3	27[1]
V	6	0	0	0	0	6
VI	7	0	0	3	0	10
VII	28	38	44	3	0	113
VIII	11	0	0	0	0	11
Total strains recovered and tested						315

[1] Includes strains clustering with at least one inoculant strain

Clustering of the rhizobia recovered from *Dalea purpurea* based on inoculant treatment and BOXAIR-PCR is also shown as an MDS Scaling plot in Fig 5.6. Notable is that all the strains recovered from the plots in which inoculated wheat was used as a cover crop cluster to a single group and coincide with inoculant strains UMR6808 and UMR7205, whereas the rhizobia recovered from the uninoculated and granular clay plots cluster into two quite distinct groups.

Unexpectedly absent in this evaluation are rhizobia from *Amorpha* and *Astragalus:* these organisms are able to nodulate *Dalea,* and have been recovered from *Dalea* trap hosts in previous studies, especially where only limited numbers of *Dalea* rhizobia were available. Their absence in third year testing here must be regarded as promising. It also appears from the PCR data that there were differences in the contribution of the different *Dalea* inoculant rhizobia, and that this was perhaps affected by inoculant formulation. This could perhaps influence the rhizobia to be recommended as inoculants in future years. To examine the importance of inoculant-strain nodule occupancy and potential for nitrogen fixation in the prairie system, we selected 55 rhizobia from those trapped on *Dalea purpurea* and derived from different diversity clusters and inoculation treatments, then evaluated these for differences in their contribution to the growth of *Dalea purpurea* grown in N deficient media in Magenta units (Tlusty et al., 2004). A very strong correlation between nodule number and plant growth on the nitrogen deficient sand/sunshine mix No 2 medium was evident (r^2= 0.595), with plants at harvest differing in fresh weight from only 22 to 345 mg per plant. For the 20 strains tested having homology to the inoculant rhizobia, plant weight averaged 130.6 mg per plant; for the 34 strains classified as different from the inoculant strains, average weight per plant was only 64.9 mg. This highlights the importance of inoculant strain quality in inoculant contribution to the developing prairie.

A total of 241 strains were recovered from the different prairie plots using *Desmodium canadense* as trap host. These were not uniformly distributed among the inoculation treatments but reflected the greater number of nodules recovered from the soil-applied inoculant and cover crop inoculation treatments. From these strains we could distinguish 32 clusters of which only two (13.68% of the strains examined) included strains clustering with any of the slow-growing *Bradyrhizobium* inoculant strains used in this study (see Figure 5.7). Interestingly these strains were not those intended for *Desmodium canadense* per se¸but were strains identifying with the inoculant strains used for *Chamaecrista fasciculata*, the legume host most abundant in the prairies in the first season after establishment. This raises several questions 1) is it better in a prairie environment to supply different rhizobia for each of the legumes that use bradyrhizobia as their microsymbionts, or to supply just one or two strains that on average will work reasonably well with all; 2) how important are the first season legumes such as *Chamaecrista* in dictating inoculant strain establishment in soil, and 3) how is it that a relatively low inoculant strain recovery rate was found, but most of the nodules collected were from the plots receiving relatively high inoculation rates. If nodulation of *Desmodium* was due to indigenous rhizobia they should have been recovered with equal frequency from all plots, including the control and seed inoculated treatments. This would suggest genetic rearrangement among bradyrhizobial strains in their three years in the soil, a phenomenon that was unlikely to have been detected with the *Dalea* rhizobia because of the genetic similarity of the inoculant strains used for this host.

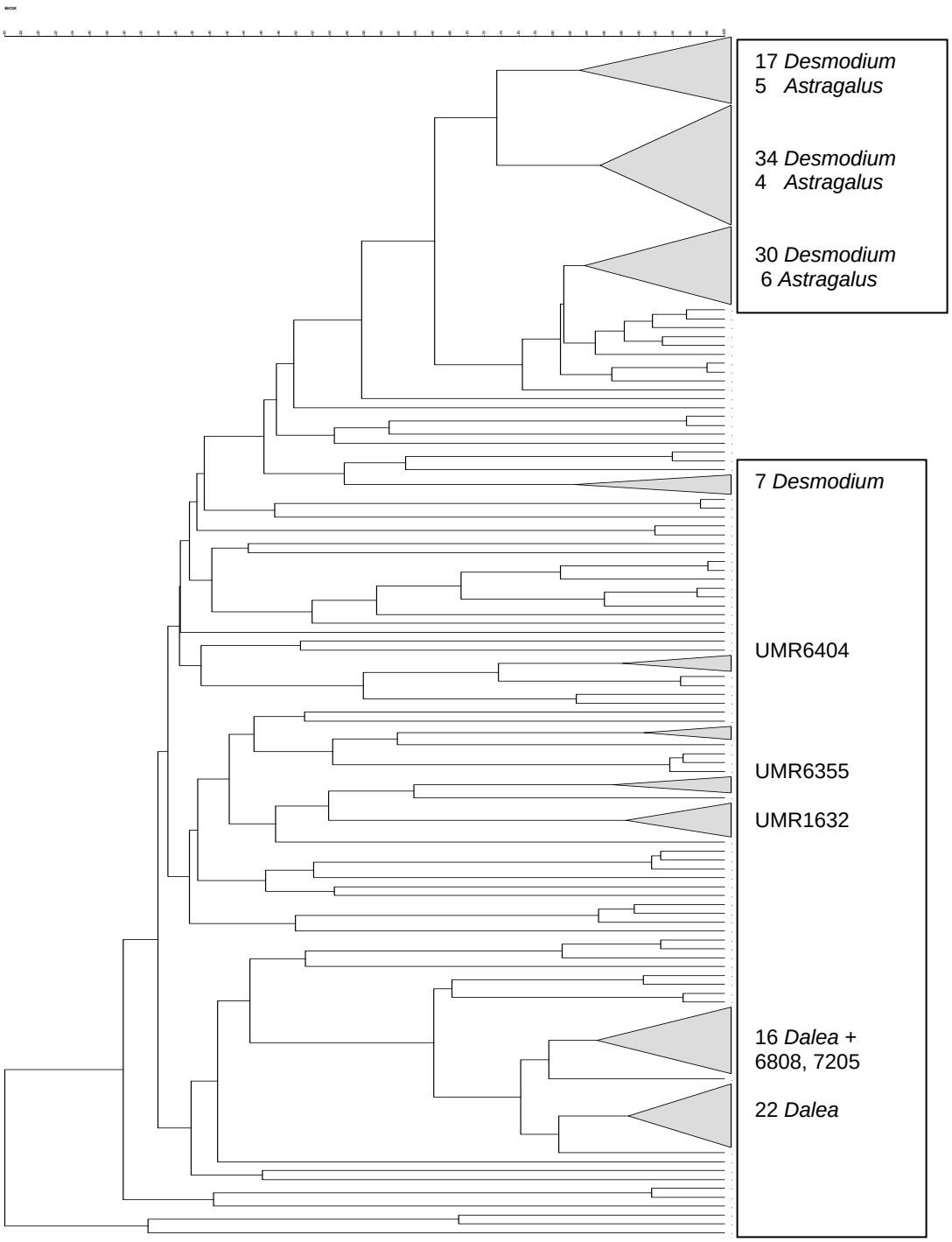
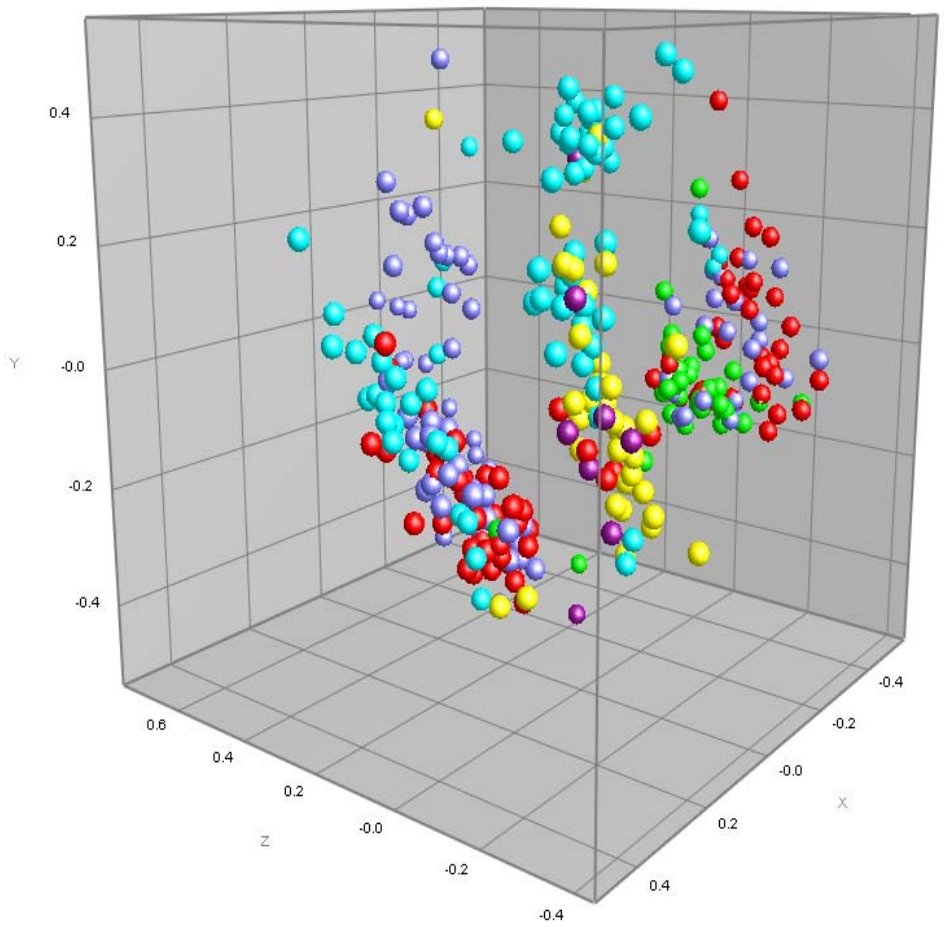


Figure 5.5. Dendrogram showing the clusters of rhizobia derived from the prairie plots in 2006 at Becker MN. Rhizobial and bradyrhizobial inoculant strains used in this study are also shown as a guide to inoculant strain nodule occupancy. Strain UMR1632 is included as a reference in all gel electrophoresis runs.



Rhizobia recovered from:

- Wheat
- Granular Peat
- Granular clay
- Seed inoculated
- Uninoculated control
- Reference inoculant strains

Figure 5.6. MDS scaling of rhizobia from *Dalea purpurea* recovered from Becker prairies three years after establishment and inoculation of the prairies using different inoculant methodologies.

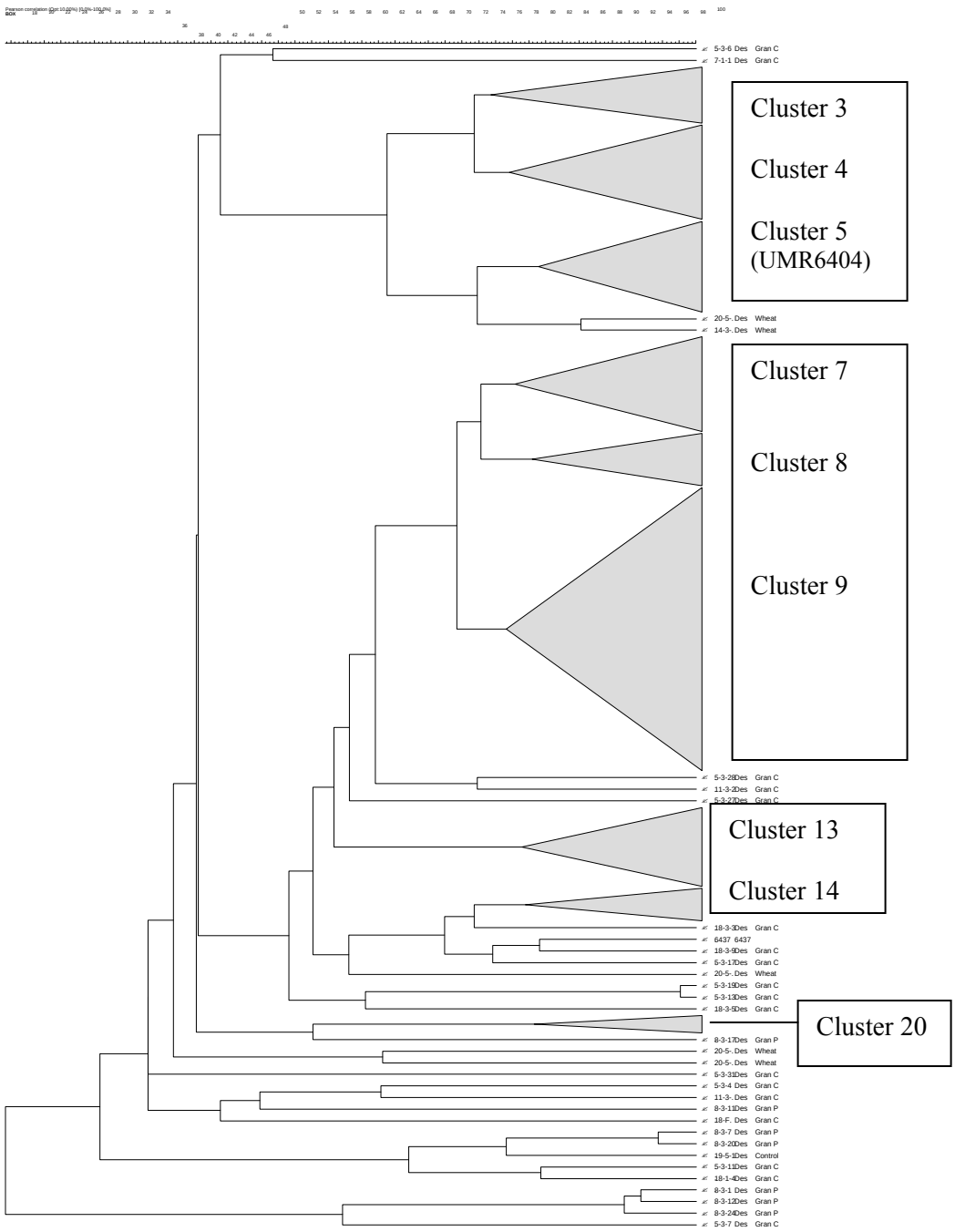


Figure 5.7. Dendrogram showing genetic similarities among the bradyrhizobia recovered using *Desmodium canadense* as trap host. Thirty-two clusters were identified among the 241 strains tested, with clusters 5 (UMR6404) and 14 (UMR6437) those that identified with inoculant strain.

Seventy-five of the nodule isolates obtained from *Desmodium* were plant tested with this host using sterile magenta units for control of contamination. Again there was a good correlation between plant dry weight achieved and nodule number (Fig. 5.8, $r^2 = 0.4679$), with nine of these isolates significantly better in their symbiosis with *Desmodium* than were the recommended inoculant strains used as controls. We are currently plant testing these strains with *Desmodium, Lespedeza,* and *Chamaecrista* to determine whether they might be better in host range and nitrogen fixation than the existing inoculant strains.Clearly the performance of the slow-growing rhizobial inoculants did not give as satisfactory results as those for *Dalea.*

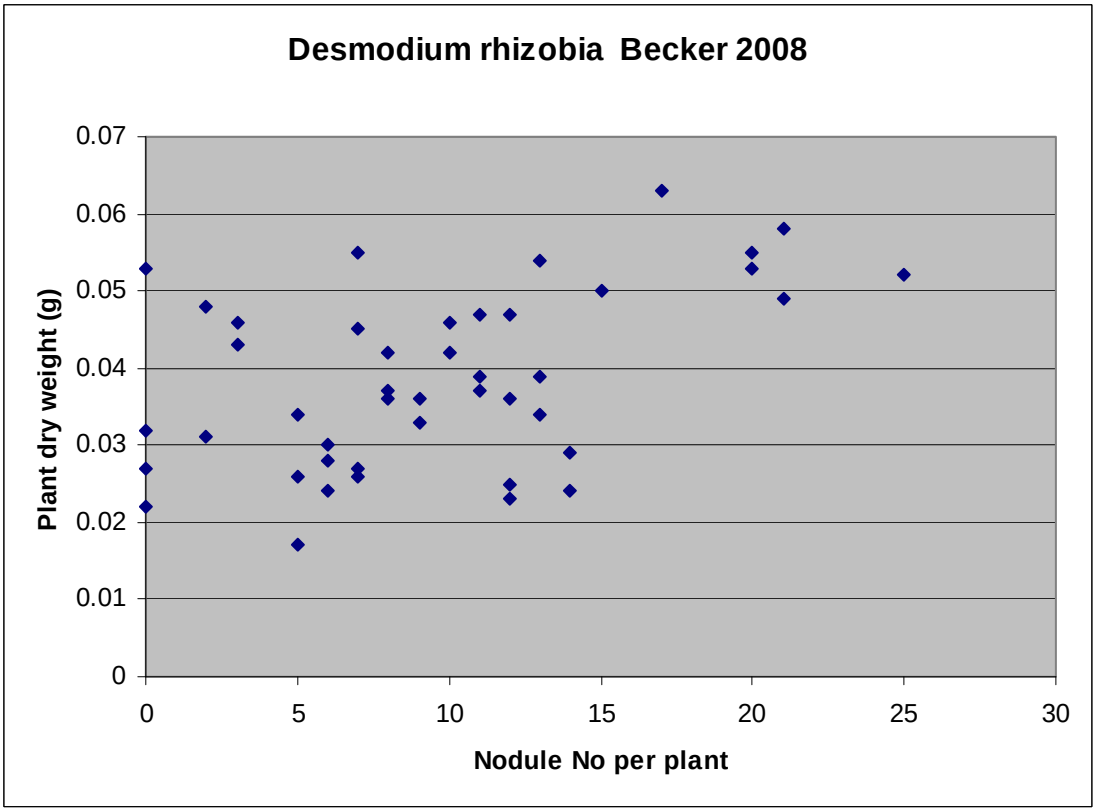


Figure 5.8 Response of diverse strains recovered using *Desmodium* used as a trap host with soils collected from the prairies in 2007, when these rhizobia were used to inoculate this host grown in sterile magenta units ($r^2 = 0.4679$).

5.4. Discussion

Norms for rhizobial strains to be used, how they might need to be varied under different environmental conditions, and an understanding of the benefits and imperfections of different inoculation methodologies are not developed overnight. That is evident in the review of the legume inoculant industry in Australia for the period 1953-2003 (Bullard et al., 2005) and in the overview of symbiotic nitrogen fixation provided by Graham (2005a). By contrast to crop and pasture systems, studies of rhizobia, legumes, inoculation practices, and nitrogen fixation in the developing and functioning prairie are in their infancy. We know little of the diversity and ecology of the N_2-fixing organisms associated with the different prairie legumes, or the rates of

nitrogen fixation they achieve, and have not begun to study the relevance of these factors to the prairie as a whole, or to restoration ecology. Methodologies that are more-or-less taken for granted in the use of rhizobial inoculants for crop species, still need to be evaluated and modified for use in the prairie. This is only the second study we are aware of to examine the inoculation and N_2 fixation of an establishing prairie, and we continue to identify more technical issues, questions of science and unexpected results, than we find answers. Even so, progress is clear, and should open the way for a diversity of studies ranging in nature from the very practical to questions of ecology and genetic interchange in soil.

In this study we tested inoculation treatments that differed in the number of organisms applied, and the inoculant carrier used, for their effects on legume establishment and contribution to a recently seeded prairie on the Anoka sandplain. For most of the prairie plant and microbial parameters evaluated over the three-year-period thereafter, we could show clear differences between uninoculated or inoculated-prairie-seed- treatments and those treatments in which granular inoculants or inoculated winter wheat seed were used. Granular inoculants supply greater numbers of soil-applied rhizobia, inoculation of the larger winter wheat cover crop allows more rhizobia to be applied per seed, and the growth of these organisms in the wheat rhizosphere before the first hard frost. Increasing the number of rhizobia applied clearly benefited prairie establishment and function. Thus in 2007 microbial biomass N (ppm), legume number per prairie, percent inoculant strain nodule occupancy for *Dalea,*and total N were 6.04 ppm, 68.0, 2 and 3.90g m^{-2} for the seed inoculated plots, but an average of 9.08 ppm, 108.9, 81.7 and 4.42g m^{-2}, respectively for the other three inoculation treatments. Importantly, when we trapped *Dalea* rhizobia from the inoculated three-year old prairie and separately inoculated each onto *Dalea* plants growing in Magenta units, plants inoculated with isolates identifying as similar to the inoculant strains produced an average of 130.6 mg per plant, whereas those inoculated with isolates lacked similarity to gained less than half that weight. Each of the different granular soil or wheat inoculation treatments had points in their favor. For inoculated wheat, high % nodule occupancy was achieved with *Dalea,* while soil microbial biomass N showed marked short-term increase; for the granular clay treatment both legume numbers per plot and legume biomass were enhanced, while the % *Desmodium* plants nodulated during host plant trapping was increased. Despite this, and mainly for its simplicity in use, we would recommend granular peat inoculation. For all of the traits studied results were toward the high end of our expectations.

There are still many points to be followed up in these studies. We need to determine actual rates of N_2 fixed in such prairies, the rate at which this is passed to associated grasses and forbs, and whether there is an optimum above which N availability could impact plant diversity. Possible fixation rates could be as high as 173 kg N ha^{-1} $year^{-1}$ (Brye et al., 2002). While this high a level of N_2 fixation seems unlikely, we do need to know more of the build up in soil N over time, and the pools in which it occurs, and how this could affect prairie composition and require management. How will the legume(s) respond to this accumulation…nodulate or fix less, or accept less effective rhizobia. Further, and because the restoration ecosystem is unique in the number of rhizobia and legumes it brings together, we need better understanding of rhizobial community structure, spatial distribution, the genetic stability of the inoculant rhizobia (Nandasena et al., 2006,2007), and the factors contributing to host-strain compatibility and inoculant strain balance over time. Brye et al. (2002) suggest that a period of at least 5 years is the minimum needed to measure restoration progress. There is clear evidence of this in differences in soil microbial biomass N between the best of the three year old prairies and that achieved in prairies seeded in 1999 and 2000 (Graham et al., 2004). Because of this we need to

revisit these prairie areas over time and to compare both soil properties and inoculant strain competitiveness. Finally we need the capacity to better compare rhizobia in the soil with those associated with their respective hosts; a process that hopefully will be facilitated by the *Rhizobium/ Bradyrhizobium* selective media being developed.

CHAPTER 6: Inoculation of prairie legumes used in seed blankets.

6.1. Background

Pre-vegetated blankets are available in Minnesota for use in shoreline stabilization, construction sites, and wetland or prairie revegetation. In order to achieve significant growth before transfer to the field, blankets are intensively fertilized, including levels of nitrogen that are likely to be inhibitory for nodulation (Gibson and Harper, 1985; Caballero-Mellado and Martinez-Romero, 1999; Dianda and Chalifour, 2002; Gan et al., 2002) and to influence plant community dynamics in favor of faster growing species (Wedin and Tilman, 1996; Baer et al., 2004; Rowe et al., 2006; Clark and Tilman, 2008). A greenhouse experiment was carried on to test nitrogen rates and time of application for their effect on legume nodulation, establishment and productivity following rhizobial inoculation.

6.2. Methods

To simulate commercial production methods for pre-germinated seed blankets [Hild & Associates Inc. (River Fall, WI)], tall grass-mesic prairie mix seeds (Prairie Moon Nursery Inc., Winona, MN) were seeded into 0.45 x 0.35 x 0.15m plastic trays, containing three layers of material, as follows:

<> an 0.05 m bottom layer of Turface MVP™ calcined clay granules (Profile Products LLC, Buffalo Grove, IL), overlain by

<> a coconut fiber blanket C125BN (North American Green, Evansville, IN)

<> a 0.025m packed layer of 3:1 silica sand and N-free Sunshine Mix 2 (Sun Gro Horticulture Inc., Bellevue, WA).

Seeding rate was 5-fold that normally recommended by Prairie Moon to ensure satisfactory plant stands, and supplemental legume seeds were pre-germinated and planted in each tray. Treatments applied are indicated in Table 6.1, with all treatments inoculated with a mix of rhizobia appropriate to the legumes planted (Tlusty et al., 2004), and designed to supply 10^3 cells of each per g topsoil. Each treatment included four replicates, with the plants grown in the greenhouse for eight weeks before sampling. Plants were watered on alternate days with modified Summerfield nutrient solution (McDermott and Graham, 1990) amended with varying nitrogen concentrations as shown in Table 6.1.

Table 6.1. Inoculation and N supply timing in blanket inoculation experiments.

Treatment	Inoculation	Nitrogen supply
1	In week 1	1.5L of 10 ppm N per week
2	In week 1	1.5L of 30 ppm N per week
3	In week 5	1.5L of 50 ppm N/week until week 5 from then on 1.5 L 10 ppm N per week
4	In week 1	1.5L 50 ppm N per week

Plant community development in the trays was studied by determination of per guild and total plant number as well as shoot dry biomass per guild (grasses, forbs, and legumes). Performance of the legumes *Astragalus canadensis*, *Dalea purpurea*, and *Desmodium canadense* was studied in more detail, with number of nodules per plant and plant shoot dry weight also determined.

To determine whether infusion with rhizobia, *Azospirillum* or *Bacillus* could be used to enhance the germination of plants identified by Prairie Moon (2008) as needing moist/cold stratification, we immersed seeds of *Asclepias syriaca* (common milk weed), *Lupinus perrenis, Baptisia australis, Chamaecrista fasciulata* and *Amorpha canescans* for 6 hours in cultures of UMR6808, UMR6437, *Azospirillum spp.* and *Bacillus subtilis,* each at a concentration of 10^6 cells mL^{-1} . The infused seeds and a seed treatment soaked in water for the same period of time, were then sown in soil trays, with 30 seeds per tray and three replicates of each seed species x inoculant treatment. Percentage germination was determined after 10 days at 28°C.

6.3. Results

Mean plant counts per guild are shown in Figure 6.1, with the only difference evident that of low legume numbers in the treatment receiving only 10 ppm N. This is a level of N sometimes recommended for legumes totally dependent on N_2 fixation for growth, and as a starter to stimulate early growth and development (Clayton et al., 2004; Osborne and Riedell, 2006). It was not adequate under conditions where the grass species showed rapid early development. As expected, shoot biomass of both forbs and grasses increased with N supply (Figure 6.2), but legume biomass was only significant in treatment 2 (1.5 L 30 ppm N per week). Nodulation was also greatest for all three legumes tested at this level of N supply, and was markedly inhibited at the 50 ppm N level (Figure 6.3), nodule number per plant declining from 10.7, 3.4 and 14.4 for *Astragalus, Dalea*, and *Desmodium* respectively in treatment 2, to 1.5, 1.4 and 1.9 nodules per plant in treatment 4.

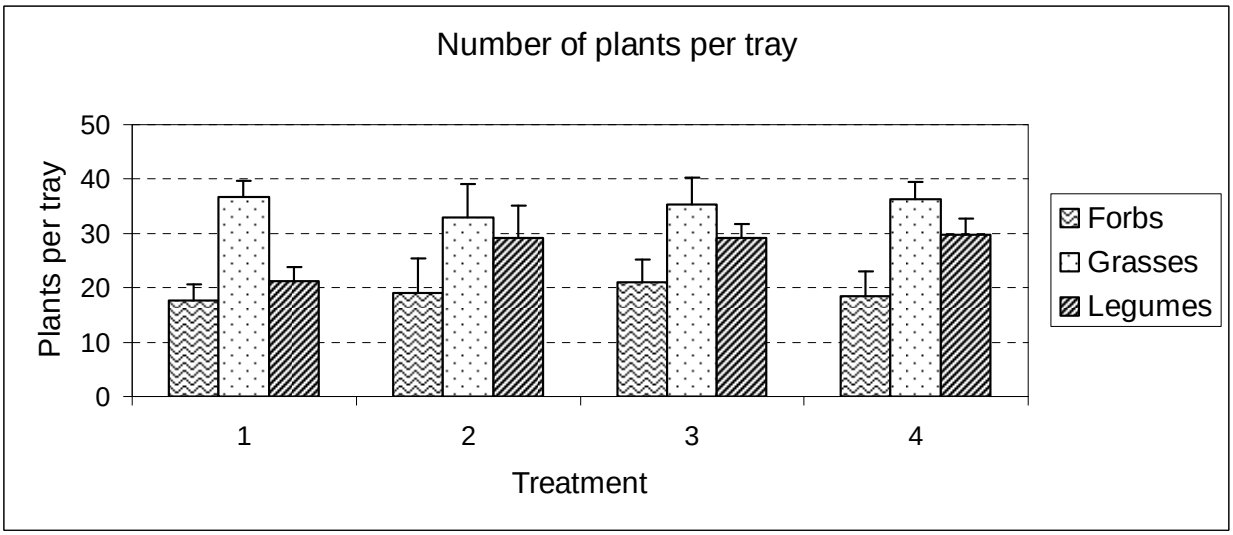


Figure 6.1. Mean per guild plant counts obtained using greenhouse grown seed blankets, 2007.

Thirty ppm N is a level greater than that normally considered inhibitory to nodulation, and again indicates the strong competition from grasses and forbs for this nutrient. Initial high N level with inoculation delayed until week 5, as practiced in treatment 3, was intended to allow early plant development in all species, but then to favor legumes through reduced N supply and inoculation at week 5. It may have been that harvest after eight weeks did not allow sufficient time for increased nodulation and N_2 fixation. Total nitrogen supplied during the eight weeks

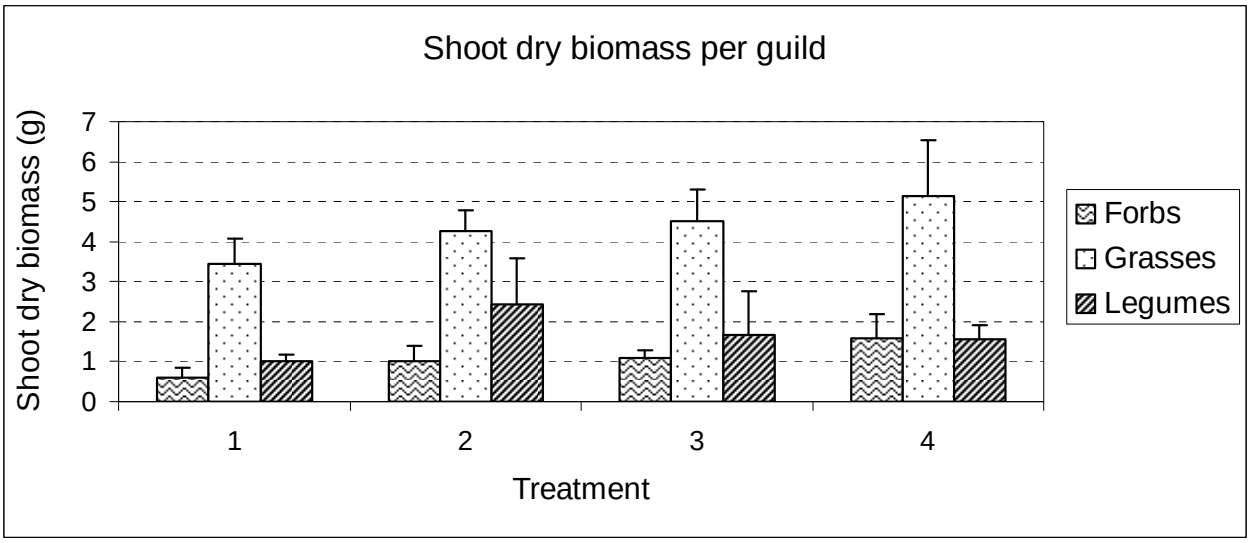
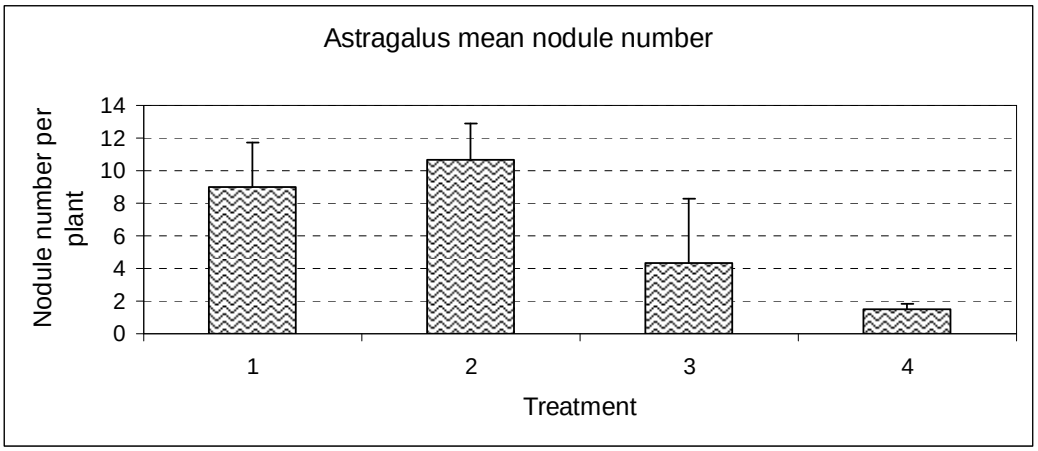
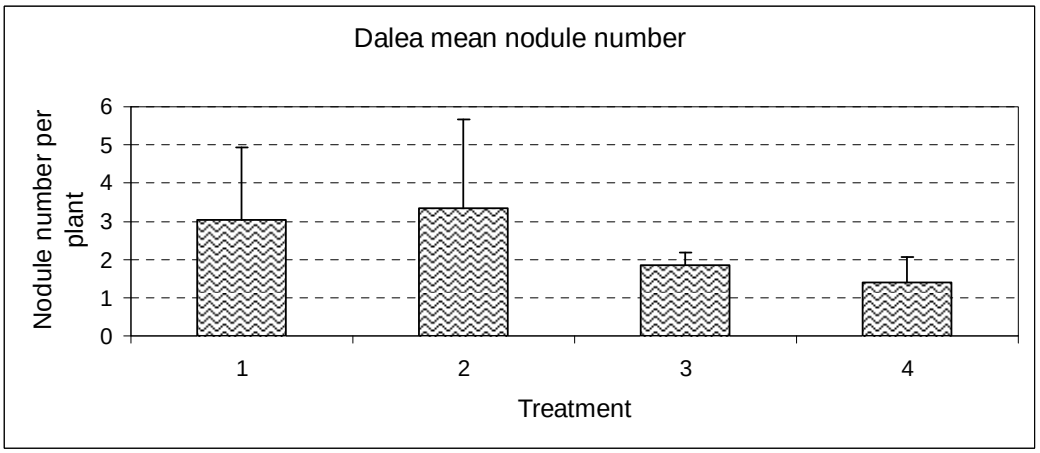


Figure 6.2. Mean shoot dry biomass per guild obtained using greenhouse grown see blankets, 2007.

experiment did not exceed 2.6 kg ha^{-1}. By the most conservative assessments, these figures fall short compared to the fertilization rates of the 47.25 kg ha^{-1} recommended by Hild & Associates, Inc. (Joel Tallaksen, pers. comm.) and, since nodulation was already affected, suggest that inoculation of the seeding blankets would not be practical. Follow up studies need to consider what happens to the legumes once these blankets are deployed in the field and the viability of inoculants over time in the blanket material or associated with seeded species. One possible benefit of the latter approach could be that added inoculants might stimulate germination and early seedling vigor in a range of species used in the blankets (Chen et al., 2007) while also contributing to subsequent legume nodulation.

No differences in germination as a result of infusion with different rhizobia or PGPM could be shown.





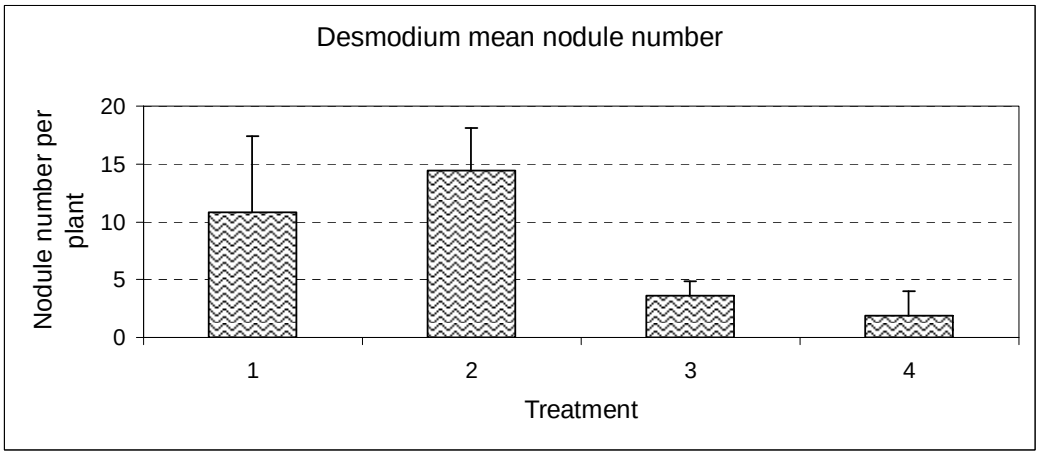


Figure 6.3. Mean nodule number with treatment, Greenhouse 2007.

CHAPTER 7: Recommendations

1. We recommend that inoculation with granular soil-applied peat inoculant be incorporated as a standard practice in MNDOT prairie seeding and restoration activities. There is a clear case in the results presented here for the need to inoculate *Dalea purpurea* and *D. candida*; though results with *Desmodium* are somewhat equivocal. While Fall-planting is commonly favored as promoting diversity in the resultant prairie, we would recommend that inoculation, wherever possible, be carried out early the following spring. This is less problematic for the rhizobia, than their overwintering near the surface of the soil, but we can accept that manpower issues may be a constraint in implementation of this recommendation.
2. In agricultural situations granular soil applied inoculants are used at the rate of 5-10 kg ha^{-1}. While lower rates of inoculation could be acceptable for early spring treatments, this would still represent a substantial increase in the amount of peat needed for restoration activities. The peats used in this project were provided by Becker-Underwood, Saskatoon, at least in part because of technical advice given them by the PI. MNDOT needs to develop a plan to cover its peat/inoculant requirements, and this should include evaluation of different peats available in Minnesota. A number of horticultural peats are available in Minnesota, and might be suitable for use in inoculant production. We are arranging to test these but will need to do so outside the present grant.
3. Additional studies need to be undertaken that determine rates of nitrogen fixation in prairie ecosystems, and the variation that is likely, as well as how this rate will affect species balance both above ground and in the soil. In cropping systems where much of the N fixed is removed in the grain, N_2 fixation must be maximized to meet seasonal plant needs and to maintain long-term soil N levels. It may well be that the best N_2-fixing strains are needed in the prairie; it is likely however that legumes will need to respond differently as the prairie matures, or when there is fire. Management strategies need detailed evaluation.
4. Studies undertaken to date explore only the major attributes of chosen inoculant rhizobia. If prairies are to persist and fulfill their function, we need to know the impact of environmental factors on nodulation and N_2 fixation, and understand the likelihood of genetic change and host swapping among the rhizobia used as inoculants. We also need to study in more detail the factors contributing to inoculant strain persistence in soil and long-term contribution to soil physical and chemical improvement.
5. Results obtained with the selection of inoculant strains for *Amorpha* and *Desmodium* to date have also been less than ideal. More work needs to be done with these species, as well as with inoculant strains for legumes important in other regions of the state. With sourcing of legume seed an issue with many agencies, we need to also understand variation with the rhizobia with which these hosts associate.

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