2004-32

Final Report

INOCULATED LEGUMES AND REVEGETATION/ROADSIDE PLANTINGS

Research

Technical Report Documentation Page

1. Report No.	2.	3. Recipients Accession No.
MN/RC – 2004-32		

4. Title and Subtitle	5. Report Date
INOCULATED LEGUMES AND REVEGETATION/ROADSIDE PLANTINGS	June 2004
	6.

7. Author(s)	8. Performing Organization Report No.
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9. Performing Organization Name and Address	10. Project/Task/Work Unit No.
Department of Soil, Water, and Climate University of Minnesota 439 Borlaug Hall 1991 Upper Buford Circle St Paul, MN 55108	11. Contract (C) or Grant (G) No. (c) 74708 (wo) 178

12. Sponsoring Organization Name and Address	13. Type of Report and Period Covered
Minnesota Department of Transportation Research Services Section 395 John Ireland Boulevard Mail Stop 330 St. Paul, Minnesota 55155	Final Report
	14. Sponsoring Agency Code

15. Supplementary Notes

http://www.lrrb.org/PDF/200432.pdf

16. Abstract (Limit: 200 words)

In response to the vast loss of prairie area in western and southern Minnesota and the concern over plant genetic loss and soil erosion that resulted, this study set to determine the success of prairie reconstruction along Minnesota roadways. Areas around rest stops on major highways were seeded with prairie plants. As nitrogen is commonly limited in natural environments, legumes- known for their ability to fix nitrogen in symbiosis- were included in the prairie reconstruction plant mixes. However, not all of the land area contains sufficient rhizobia to maintain the legumes. Therefore, inoculation with rhizobia was necessary. The predominate goal of this study was the establishment, inoculation and continuing observation for traits associated with nodulation and nitrogen fixation of prairie lands of different ages at the University of Minnesota Sandplain Experiment Station in Becker, Minnesota. The area was then monitored for plant development and survival

17. Document Analysis/Descriptors	18.Availability Statement
Legumes Nitrogen fixation Rhizobia Re-vegetation	No restrictions. Document available from: National Technical Information Services, Springfield, Virginia 22161

19. Security Class (this report)	20. Security Class (this page)	21. No. of Pages	22. Price
Unclassified	Unclassified	62	

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Final Report

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June 2004

Published by:

Minnesota Department of Transportation
Research Services Section
395 John Ireland Blvd Mail Stop 330
St Paul MN 55155-1899

ACKNOWLEDGEMENTS

The authors thank the members of our Technical Advisory Panel for their assistance and support in this project. Members from the Minnesota Department of Transportation (MNDOT) included Bob Jacobson, Dwayne Stenlund and Jim Klessig. We also appreciate the help we received at our Becker field site from Glenn Titrud and Ronnie Faber.

We want to acknowledge the support of Catherine Brown, Ryan Vial, Maina Martir, James Eck, Dan Lynch and Evan Cree in the execution of this research; of Roger Eliason and the staff of the Plant Research Analytical Laboratory for soil and plant analysis; and of Keith Piotrowski for soil quality traits. Thai Doan worked on the winter wheat inoculation project during a fellowship from the Minnesota Academy of Science in 2003.

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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 of the prairie area and the removal of ground cover has raised concerns of plant genetic loss and soil erosion in the area, and has spurred efforts at reconstruction. In the case of the Minnesota Department of Transportation (MNDOT) this includes designation of grassland areas alongside roadways as "Wildflower Routes", the seeding of areas around rest stops on the major highways with prairie plants, and extensive restoration for wetland mitigation. A concern in establishing these areas is that they provide an attractive environment needing only limited attention post-establishment.

Nitrogen is commonly a limited nutrient in natural environments, and if these prairie areas are to be sustainable, a system must be developed that maintains 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. While this is routine for agricultural legumes, the successful inoculation of prairies or prairie legumes with an appropriate strain(s) of rhizobia presents problems not encountered in agricultural situation. 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 planting; low seeding rate for the legumes used, and irregular germination of these legumes in the following season, with limited opportunity for build up of rhizobia in soil.

The main thrust of the study reported here was the establishment, inoculation and subsequent monitoring for traits associated with nodulation and nitrogen fixation of prairie areas of different ages at the University of Minnesota Sandplain Experiment Station, Becker MN. Prairies planted using a shortgrass prairie mix containing the legume species *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea candida* and *D. purpurea, Desmodium canadense* and *Lespedeza capitata* were seeded in 1999, 2000 and 2002. Each area seeded was heavily inoculated with a mix of rhizobia identified as being of inoculant quality for the legumes in question; in the planting in 2000 some areas of prairie were not inoculated. Each area was monitored for plant and species development, for inoculant survival and for traits associated with nitrogen fixation. In July 2003 all areas were sampled at the same time, with data collected on species establishment, grass, forb and legume biomass production and N content; soil physical, chemical and biological properties; rhizobial persistence in soil and beneath plants, and percentage of nitrogen derived from fixation.

Even though the prairie areas were mowed annually and raked to induce nitrogen deficiency, legume establishment in the prairie areas was slow. However by the end of the study the 1999 prairie was legume and grass dominant, and the 2000 prairie well established. N accumulation though nitrogen fixation had begun to occur and soil biology had begun to reflect N accumulation. Surprisingly, when soil samples were collected

from random sites in the 1999 and 2000 prairies, few samples had significant counts of rhizobia, even though legumes in the prairie were deriving a significant percentage of their N through nitrogen fixation. A much higher percentage of samples taken from beneath legume plants contained rhizobia suggesting, perhaps, that rhizobia do not survive well in the soil away from their host, and that their numbers could be negatively impacted by the prolonged period between seeding and active legume growth. Contrary to this view, a high percentage of the rhizobia for *Dalea* and *Astragalus* recovered from the older prairies had the same genetic fingerprint as the inoculant strains. It was surprising that while the inoculant strain for *Amorpha canescens* could not be recovered from soil with *Amorpha* as trap host, a high percentage of the nodules formed on this host had the genetic fingerprint of the inoculant rhizobia for *Dalea.* As an alternate approach to conventional methods of inoculation, we tested the application of inoculant to a cover crop such as winter wheat. While more work needs to be done, initial experiments were successful.

Conventional inoculation wisdom is that *Chamaecrista, Desmodium* and *Lespedeza* belong to the so-called cowpea miscellany, and are broadly promiscuous, whereas *Amorpha*, *Astragalus* and *Dalea* are specific in their rhizobial requirement**.** This was not evident in a study where rhizobia were trapped from the soils of the Hayden, Kellogg/Weaver Dunes and Ordway prairies using *Dalea purpurea* as host and characterized using genetic fingerprinting and 16S rRNA sequence analysis. In a study in which a total of 211 strains were tested, most of the isolates from Hayden prairie appeared similar to *Rhizobium etli*, most from Ordway to *R.gallicum* and most from Kellogg/Weaver Dunes to either *M. huakuii* or *M. amorphae.* Isolates with characteristics similar to *Mesorhizobium amorphae* were also recovered from *Dalea* nodules in this study. It would appear from this that transfer of rhizobia could occur among hosts in the prairie environment, and if these strains are not equally effective in symbiosis with their new host could perhaps affect long-term benefits to the prairie. More studies need to be done to determine the frequency of strain recovery from alternate hosts, and the effect of strain transfer on symbiotic nitrogen fixation. The decision by the University of Minnesota to maintain these prairie areas beyond the life of this project will provide opportunities for longer-term studies and more detailed comparisons over time.

Chapter 1. Introduction

Prior to settlement, more than 18 million acres of western and southern Minnesota were covered in prairie; today less than 0.5% of that prairie area remains (1). Much of this residual area is inaccessible to farm machinery, occurs along roads and rights of way, and is in various stages of degradation. Fragmentation of the prairie areas has raised concerns of genetic erosion and loss and has spurred efforts at restoration. The Minnesota Department of Transportation is one of many organizations participating in this effort, designating research funds through its Office of Environmental Services for research on restoring and retaining prairie areas. MNDOT is responsible for the maintenance of grassland areas alongside roadways, and has designated a number of these (for example highway 10 near Becker, MN) as "Wildflower Routes". Areas around rest stops on the major highways are also commonly planted with prairie species, as a means of providing an attractive environment needing only limited attention post-establishment.

A major concern in these areas is that of nutrient supply. Nitrogen is commonly a limiting nutrient in natural environments, and its shortage exacerbated by fire and grazing. If the prairies are to be truly sustainable, as were the natural prairies that were once so dominant in this region, a system must be developed that maintains soil nitrogen at levels adequate for prairie plant growth and for the ground cover needed to minimize erosion and degradation of roadside and other restoration areas. Legumes, because of their ability to nodulate with rhizobia and to fix nitrogen in symbiosis, have long been recognized for their importance in natural ecosystems. Fixation rates in such ecosystems vary but can reach more than 50 kg ha^{-1} under managed conditions. Because of this MNDOT includes a number of indigenous legumes in the mix of plants used for roadside revegetation and wetland reconstruction activities. These include *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea purpurea* and *D.candida, Desmodium canadense* and *Lespedeza capitata.* Unfortunately, not all of the land areas under revegetation contain rhizobia suitable for symbiosis with these legume species. Where this problem arises in an agricultural situation, it is usually easy to obtain commercial preparations of the appropriate rhizobia, and to inoculate these into the soil. Where this has been done commercially, the inoculated organisms may still persist in the soil 10-15 years after inoculation. Unfortunately, the successful inoculation of prairies or 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 of the information dates to the studies of Bushnell and Sarles (2); Shave and Pengra (3) and Falken and Pengra (4), and many of the organisms used 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 often exposed near the surface over the subsequent winter period
- Rate of seeding for each legume used in prairie restoration or roadside revegetation activity is usually low, limiting the number of rhizobia that can be applied through the traditional method of seed inoculation. This limits rhizobial survival during overwintering, and affects subsequent nodulation and nitrogen fixation.

● Several of the legumes sown have distinct vernalization requirements with germination dependent on seed scarification or moist/cold stratification. Even when these treatments have been applied, seed germination may still be delayed and irregular.
● In the agricultural situation the norm is that a single plant species is sown, usually at densities exceeding 100,000 plants ha^{-1}. During the growing season more than 25 kg ha^{-1} of nodules may be produced, and when these senesce 10^9 rhizobia g^{-1} of nodule tissue may be released back into the soil. This acts as an inoculant for future crops of that species, and in most circumstances means that inoculation need only be practiced in the first year. In the "prairie" environment, legume plants are more widely spaced and the build up of rhizobia in soil following nodule senescence is limited. Furthermore, because multiple legumes are seeded, and several of these may be able to nodulate with a number of different rhizobia, but not necessarily to fix nitrogen during symbiosis, the process under prairie conditions may be much less efficient than in the agricultural environment.

The main goal of this project was to use rhizobia that had been selected for their ability to nodulate and fix nitrogen in symbiosis in the inoculation of areas designated for revegetation and prairie establishment, and to follow the effect of inoculation on subsequent prairie development and soil change. Because a number of the tasks associated with this project involved the provision of information for the better utilization of rhizobial inoculants, we also provide a significant amount of background information on the process of strain selection and testing, inoculation methods, and the measurement of traits associated with this process.

The information in this report is divided into 7 chapters. In Chapter 2 we provide an overview of inoculation, nodulation and nitrogen fixation, considered from a prairie perspective. Chapters 3 and 4 describe establishment of inoculated prairies of different ages at Becker and Stillwater, MN, and reports on changes on plant growth, development, nodulation and nitrogen fixation associated with inoculation of the prairie legumes involved. Chapters 5 and 6 consider some alternate methodologies for prairie legume inoculation and the inoculant needs of this industry. Chapter 7 reports a study to examine differences in the rhizobia associated with the prairie legume *Dalea purpurea* in four prairie areas in Minnesota and Iowa, and considers possible impacts of fragmentation on *Rhizobium* diversity.

Chapter 2: Overview of inoculation, nodulation and nitrogen fixation: a prairie perspective

What is nitrogen fixation and how is it carried out

Nitrogen fixation is a process by which nitrogen gas from the atmosphere is converted to ammonia. It can be an industrial process (as in the production of nitrogen fertilizer) but in most natural systems is a process mediated exclusively by specific groups of prokaryotic organisms. All possess the enzyme nitrogenase which actually undertakes fixation of the nitrogen. These nitrogen-fixing prokaryotes can exist as free-living independent species, for example in soil, or can be associated with other specific microbes, plants or animals. Not all prokaryotes can fix nitrogen, and in those that do, rates of nitrogen fixation can vary very markedly. Since nitrogen fixation is an energy requiring process, the availability of a suitable energy supply is the factor most commonly limiting rates of nitrogen fixation. A consequence is that where the organism is living free in the soil and competing with many other microbes for available carbohydrates, rates of nitrogen fixation are commonly of the order of only 10-15 kg ha^{-1}. In contrast, in the specific association of legumes and rhizobia, the bacteria fix nitrogen from a form the plants cannot use into one readily assimilated by their host, and in return are provided both a specialized structure (the nodule) in which to live, and abundant photosynthate from their host on which to grow and fix nitrogen. Rates of nitrogen fixation in commercial crops such as soybean can be as much as 400 kg of nitrogen ha^{-1} $year^{-1}$, and provide essentially all of the nitrogen that the legume host needs for growth. There is no comparable data for the contribution of legumes to the nitrogen needs of the establishing prairie, but as with other grass/legume mixtures, shading from the grass will reduce fixation, while shortage of N will favor legume development. The result is likely to be that fixation is less than achieved in pure stands of the legume, while a significant part of the nitrogen fixed ends up in the grass or other forbs present.

Bacteria capable of nodulating and fixing nitrogen in association with legumes include both α- and β proteobacteria, the latter only recently identified as symbiotic with legumes, and still represented by very few species (5, 6, 7). The α proteobacteria forming nodules on legumes are referred to generically as rhizobia, and are currently divided into six genera, *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium*, with 38-40 species. Legume hosts and corresponding bacteria that are important in the Minnesota prairie, and utilized by MNDOT, include:

Amorpha canescens.....Mesorhizobium amorphae (8)
Astragalus canadensis.....Mesorhizobium huakuii (9, 10)
Chamaecrista fasciculata.....Bradyrhizobium spp. (Chamaecrista)
Dalea purpurea, D.candida and D.villosa.....Rhizobium spp. (Dalea)
Desmodium canadense.....Bradyrhizobium spp (Desmodium)
Lespedeza capitata....Bradyrhizobium spp. (Lespedeza)

Note here that several of these organisms have not been characterized beyond genus level. They are identified by the genus of bacteria to which they belong, and by the host from which they were isolated.

The process of nodulation and nitrogen fixation

Rhizobia can infect their hosts and induce root- or stem-nodule formation using several different mechanisms. However the only two of these likely to occur with the prairie legumes mentioned above are:

- penetration of root hairs and formation of infection-threads, as found in plants such as clovers and beans,
- entry via wounds or sites of lateral root emergence, as found in peanut (*Arachis hypogaea*)and the pasture legume *Stylosanthes*

The former is undoubtedly more common. Infection via root hairs begins with attachment of rhizobia to immature, emerging root hairs of a compatible host. This occurs within minutes of inoculation, and proceeds until rhizobia appear to cap the root-hair tip. Deformation and curling of the root hair follows with the root hair surface at the point of infection softened and hydrolysed to permit penetration by as many as 20 rhizobia. It follows that more than one type of rhizobia may gain entry at this time. Rhizobia then move down the root hair toward the root cortex. Rhizobia do not gain direct intracellular access to their host, but as they move down the root hair remain enclosed within a plant-derived infection thread. In some *Chamaecrista*, rhizobia may never escape this containment, but no studies to our knowledge have examined this detail in *C.fasciculata.* Rhizobia are eventually released into modified cells of the root cortex, where again they are enclosed within a plant-derived membrane. These membranes protect the bacteria from the defense responses of the host.

The process of nodulation involves sophisticated interaction of host and rhizobia. This includes exudation of flavonoid compounds from the root that trigger expression of nodulation genes in the rhizobia, and the production of powerful "nod-factors" by the bacteria that condition the plant for infection by the microbe and lead to the formation of the nodule structure. Legumes vary in the type of flavonoids they produce, and rhizobia respond differently to specific flavonoids. Strains of rhizobia may produce several nod factors, all having a similar core structure, but with minor chemical differences distinguishing which plants can be nodulated.

The root nodule that forms can also differ in appearance and structure, a trait determined by the host legume. *Determinate* nodules, such as occur in *Desmodium canadense* are round and have no pronounced growing point; the nodules of *Dalea purpurea* and *Astragalus canadensis,* in contrast, are elongated with a pronounced meristematic region. In these plants the nodule can increase in length over the course of the growing season. Nodules, that are active in nitrogen fixation are usually pink or red in color and are said to be *effective*. Nodules that are limited in nitrogen fixing activity are usually white or greenish brown, and are termed *ineffective*. Nodules do not remain active for the life of the plant, but usually begin to senesce 50 to 60 days after formation, with new nodules then formed. Commonly this means that plants in the field will have more than one flush of nodule formation during the growing season. It also means that over time the newer nodules will be formed on smaller secondary roots.

Relatively few infections result in root-nodule formation. In those that do, nodules may become visible as soon as five to six days after exposure to rhizobia, and begin to fix nitrogen eight to fifteen days thereafter.

Cross Inoculation

Given the signaling that occurs between host and *Rhizobium* before nodulation can occur, it is not surprising that there is considerable specificity in the legume/*Rhizobium* symbiosis. Early studies demonstrated that no rhizobial strain or nodule isolate nodulated all legumes, but that each had a finite host range, nodulating certain legumes but not others. This led to the concept of cross-inoculation groups with legumes clustered according to the different rhizobia with which they formed nodules. More than 20 different cross-inoculation groups were soon identified, with the bacteria associated with the clover, medic, bean, lupin, pea, and soybean groups each then distinguished as separate species based largely on their host specificity. Host specificity is clearly important in the provision of suitable rhizobia for legumes in the field, with individual isolates varying from those that will nodulate over 100 species of legume (for example NGR234 (11) to those that may not even nodulate all species of legume in a given genus. As a consequence more than 100 different strains of rhizobia are needed to satisfy the inoculation requirements of currently important legume species.

Where do the rhizobia of the legumes important in the prairie situation fit in to this concept? The rhizobia isolated from *Chamaecrista fasciculata, Desmodium canadense* and *Lespedeza capitata* are all slow-growing bradyrhizobia belonging to the so-called cowpea miscellany (2, 12). All are promiscuous in the range of host plants they will nodulate, though in the case of *Chamaecrista* and *Lespedeza* this range has not been clearly defined. Sprent (13) describes the evolution of symbiosis in *Chamaecrista,* and notes the distinction between *Cassia, Senna* and *Chamaecrista,* with only the latter genus usually nodulated. She also comments on the progression in *Chamaecrista* from symbioses in which rhizobia are never released from the infection thread, to species such as *C.fasciculata* in which the indeterminate nodule structure is well developed. Burton (12) reports a study in which eight of nine isolates from *Lespedeza capitata* nodulated but did not fix nitrogen with *Chamaecrista fasciculata,* whereas most were effective in symbiosis with *Arachis, and Cyamopsis.* The same strains were highly variable in their effectiveness with different *Lespedeza* species, highlighting the difficulty of providing effective inoculant strains for all members of this genus. *Desmodium* species have a reputation for nodulating with rhizobia from numerous other legumes, but of being limited in the amount of nitrogen that is then fixed (14, 15). Specific strains are recommended for this species, but don't always compete well with the more numerous indigenous organisms in the soil. In comparison with the information available for the cowpea type rhizobia and their prairie hosts, Bushnell and Sarles (2) complained that the cross-inoculation relationships of rhizobia from *Dalea, Astragalus, and Amorpha* were difficult to study. Follow up to this initial study has been limited. One inoculant company, Nitragin, has traditionally prepared different inoculants for each species, and even distinguished between the inoculant needed for *Dalea purpurea* and *D. leporina* (16); Becker Underwood prepares a single inoculant supposedly suitable for all prairie legumes. There is considerable, but as yet incomplete evidence of cross inoculation between *Astragalus, Onobrychis, Oxytropis, Dalea* and *Phaseolus* (16, 17, 18)

Legume Inoculation

When a new legume species is introduced into a region, or where soil has been piled and stored for some time, appropriate rhizobia will be scarce or absent, and inoculation with rhizobia for the legume(s) to be sown will often be critical for adequate nodulation and N_2 fixation. Data for prairie legumes is essentially non-existent but for forages, biomass increases following initial inoculation can exceed 50%, with clear visual differences evident between inoculated and non-inoculated plants.

Inoculation in subsequent years is often not needed. In fact, where a legume has a long history of cultivation in an area, most soils will contain appropriate rhizobia, and even non-inoculated plants will be heavily nodulated. Strains used in any subsequent inoculation usually give rise to only a small fraction of the nodules formed, and a yield response is unlikely. Unfortunately, many indigenous soil rhizobia are less than fully effective in N_2 fixation.

A simple, three-treatment experiment will establish the need for inoculation:

- non-inoculated control plots,
- plots inoculated with a strain of rhizobia effective on the host legume being evaluated, and
- plots inoculated with the same strain, but also supplied fertilizer nitrogen.

Where the non-inoculated plants are extensively nodulated, the soil must already contain rhizobia able to nodulate the host plant tested, the contrast between the non-inoculated plants and those supplied with nitrogen then being a measure of the effectiveness in N_2 fixation of the indigenous rhizobia. If the non-inoculated plants are green and vigorous, inoculation is probably not necessary. Absence of nodulation in the non-inoculated plants, but extensive nodulation of those that are inoculated is an indication that inoculation is needed, with the difference in plant growth in the three treatments an indicator of the effectiveness of the inoculant strain being tested. Excellent plant growth in all three treatments shows either that the native rhizobia are highly effective and inoculation not necessary, or that the site was high in available nitrogen, while poor growth in all treatments suggests that a factor other than nitrogen is limiting plant growth.

Where inoculation is needed there are a number of approaches that can be used. These have as their goals:

- to supply adequate numbers of a rhizobial strain(s) for good nodulation and effective N_2 fixation,
- to ensure that the inoculant strain(s) form most of the nodules produced, and
- that these organisms persist in soil over time, obviating the need for reinoculation in subsequent years.

Inoculant rhizobia for most of the legumes used in agriculture are now commercially produced and packaged, and available in a range of formulations designed to meet different seeding needs. Most inoculants are still peat or clay-based, and for the more important legumes usually exist in non-sterile format, or with the carrier sterilized before the addition of culture. Many farmers are now indicating a preference for liquid inoculants because of the ease with which they can be applied. The range of products available for prairie legumes is currently much more restricted because the volume of sales is more limited, and companies less likely to undertake the research needed to back stop high quality inoculants.

The following inoculation procedures are used in association with crop plants, and have been applied with variable success to the inoculation of legumes grown in natural or revegetation settings:

Seed inoculation. The inoculant is mixed with milk or some other slightly adhesive material, and the seed uniformly covered with this suspension. The seed is dried in the shade, and ideally sown the same day. This procedure requires that the inoculant strain(s) be packaged in a relatively fine carrier material or liquid that will adhere to the seed.

Seed pelleting. A stronger sticker, such as gum arabic or methyl cellulose is used with the inoculated seed then rolled in ground limestone or rock phosphate. Pelleting combats unfavorable soil conditions such as low pH or high temperature, and is used for aerial sowing.

Soil inoculation with a granular peat or liquid. The inoculant is packaged in a coarse peat, or granular clay-based product, or as a liquid that can be spread with the seed or watered on after planting. Granular soil inoculation is less time consuming, and allows higher rates of inoculation for occasions where soil conditions are unfavorable, or delays expected between seeding and germination. It also permits separation of the rhizobia from fungicidal seed dressings and toxic fertilizers.

The simple mixing of inoculant and seeds in a planter box or seeder is not recommended. Inoculant and seed tend to separate and not all seeds receive rhizobia.

Inoculation is a numbers game having as its goal a specific number of rhizobia applied per seed or per unit area. For small seeded forage plants the goal is to provide a minimum of 10^3 rhizobia per seed, or approximately10^9 ha^{-1}. This would rarely be achieved in the case of prairie legumes, in part at least because so few seeds of each species are sown per ha, but also because many of the practices needed for effective inoculant storage and use are not carefully followed. This will be discussed in more detail in the experimental sections which follow.

Where inoculation is required, the inoculant strain(s) used must have the following characteristics (19):

- 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.

Less than 1 strain in 100 is likely to meet these criteria, so rhizobia for inclusion in inoculants must undergo extensive testing. This will usually include growth chamber and greenhouse evaluation of numerous strains followed by further evaluation of selected isolates under field conditions. Host variety x strain interactions are common, so the goal must be to identify strains that are broadly effective with the legume(s) that are to be used. Environmental factors that could influence strain performance, for example soil acidity or temperature tolerance, need to be considered.

Once a suitable inoculant strain has been identified, it must be grown and incorporated into a carrier that will allow its survival in high numbers until used. Once mixed with this carrier, inoculant preparations should maintain their viability and be warranted for a shelf life of 6-12 months. Characteristics of a good inoculant carrier are:

- high water-holding capacity,
- nontoxic to *Rhizobium,*
- available, inexpensive, and easily processed,
- sterilizable by autoclaving (pressurized steam) or preferably radiation,
- adheres well to seed, and
- good buffering capacity.

As stated above, the most commonly used carriers are peat-based, but because peat is not universally available, compost, bagasse (derived from the milling of sugar cane), coal, polyacrylamide, vegetable oils, clays and clay granules, liquid preparations, and soil have all been used successfully. Carriers that have been sterilized before the rhizobial strain is added generally support a higher population of rhizobia, and have longer shelf lives than non-sterilized materials.

Not all inoculants available on the market are of good quality. Factors that contribute to this in the case of prairie legume inoculants include:

- Inadequate initial testing in the selection of the inoculant strain, and limited follow-up research to ensure that the strain(s) maintain their effectiveness
- Poor storage and transport conditions.
- Repackaging of inoculants into smaller units for sale with seed, with inadequate attention to inoculant moisure content.

Inoculants can fail as well for reasons associated with conditions in the field. These include

- Inadequate numbers of rhizobia applied,
- Prolonged periods of exposure to cold or dessication in soil between inoculation and seed germination,
- High levels of soil N,
- Contact with fertilizers or with pesticides
- Use of inappropriate gums or stickers, and
- Use of the wrong inoculant culture.

Catroux et al. (20) in a recent review of trends in inoculant production and use concluded: "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."

Measuring success of nodulation and the contribution of the inoculant strain(s)

A number of different parameters can be used to measure response to inoculation and improved nitrogen-fixing ability, or rhizobial numbers in soil or persistence. Not all of these are equally applicable to the prairie situation. They include:

Nodule number per plant: Nodule number varies with both strain used and host genotype (21) and so can vary within quite wide limits. It is most useful as an indicator of inoculant success under conditions of adversity that might be expected 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. It is less useful under productive soil conditions or where plants are large and can have many hundreds of nodules. 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.

Nodule mass per plant: Plants regulate nodule formation, limiting further nodulation when they believe they have sufficient nitrogen for their growth needs. 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. Commonly, where the strains used are ineffective, nodule number will be high but nodule size limited. Conversely where nodules are effective there will be fewer, but larger nodules. Thus nodule mass per plant is usually directly correlated with plant growth under N-limited conditions, and an excellent indicator of nodule function. A difficulty with prairie plants is that many are deep rooted, and complete recovery of nodules without significant damage to the prairie is impossible.

Plant dry weight per plant: Legume dry matter production in plants grown under N limited condition is highly dependent on nitrogen fixation and very strongly correlated with nodule mass and nitrogen fixation. A significant percentage of that N remains underground at harvest, but in deeply rooted plants is difficult to access.

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 in further plant growth rather than accumulate in the tissues. As a result total N rather than N concentration is a better indicator of nitrogen fixation under N limiting conditions

^{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 plants such as legumes that can satisfy at least part of their nitrogen needs through fixation tend to accumulate relatively more ^{14}N than ^{15}N. This difference can be measured relative to appropriate non nitrogen-fixing controls using a stable isotope mass spectrometer coupled to a C/N autoanalyzer (22) 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. This methodology appears appropriate to legumes in the prairies, where coneflowers or grasses can be used as the non nitrogen-fixing control, but there is the risk that these explore different fractions of the soil and so differ in N accumulation. N taken up by the plant can 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.

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 (23)

Nodule occupancy and success in nodulation:

In a field test with inoculants it is very possible that soils contain indigenous rhizobia, and that these will produce some to all of the nodules that form. It follows that tests are needed to determine whether the inoculant strain(s) contributed significantly to nodule formation. A number of different approaches are possible, including:

a) Serological tests identify organisms on the basis of their reaction with specific antisera (24). Difficulties are that antisera for the organism(s) in question must first be prepared; it must be established that the soil organisms are, in the main, different serologically from those that are used as inoculants; and numbers of different serogroups per species of organism are limited.
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 (25). Difficulties are that it 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 (26, 27). Relatively simple methodology but in each case can be complicated by fact that antibiotic resistance may be accompanied by change in nodulation or nitrogen fixation traits, significantly weakening the inoculant organism. In many such studies this has not been adequately investigated.
d) Genetic fingerprinting uses a DNA primer to generate unique DNA bands, then amplifies these using the polymerase chain reaction (28). Bands are then resolved on agarose gels and viewed by staining with ethidium bromide. Typical examples of this approach are given in chapters 3 and 7. The procedure is moderately expensive to set up and run, and requires training to use, but allows quite high sample throughput.

Chapter 3: Rhizobial inoculation, nodulation and nitrogen fixation of prairie legumes used in revegetation activities in Minnesota: The Becker experience

Background

Natural ecosystems are commonly limited in nitrogen (29, 30) and, after fire or grazing, can be dependent on leguminous plants for nitrogen inputs (31). 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 including *Amorpha canescens, Astragalus canadensis, Chamaecrista fasciculata, Dalea purpurea* and *D.candida, Desmodium canadense* and *Lespedeza capitata.* The successful inoculation of these legumes with an appropriate strain(s) of rhizobia presents difficulties not encountered in a more agricultural situation. Thus:

- Information on the *Rhizobium* requirements of most prairie legumes is limited, with little advance in our knowledge in recent years. Most of the information available dates to the studies of Bushnell and Sarles (2); Shave and Pengra (3) and Falken and Pengra (4); many of the organisms from these earlier 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
- Seed numbers for each legume sown, and consequently of the rhizobia that can be supplied via seed inoculation is low
- Some of the legumes seeded can nodulate with more than one of the different rhizobia used as inoculants, but are not necessarily efficient in symbiosis with these strains: transfer of inoculant rhizobia from the host for which they were intended to other legumes in the seed mixture is a possible complicating factor; and
- Germination of the legumes sown can be delayed and irregular (32).

In this study we undertook the establishment of a short-grass prairie area containing a number of legume species on a dry-land site at the University of Minnesota Sandplain Experiment Station, Becker, MN. We paid special attention to, and monitored, the development, nodulation, nitrogen fixation, and contribution of the legume species *Amorpha canescens, Astragalus canadensis, Dalea purpurea and D.candida, Chamaecrista fasciculata, Desmodium canadense* and *Lespediza capitata* to the growth of the prairie, and to the nitrogen economy of plant and soil.

Methods

Rhizobial strain selection

Most of the rhizobia used in this study were isolates trapped from soil collected from Science Nature Areas and Nature Conservancy sites in Minnesota. Because we were limited in the number of plants we were permitted to take from these sites, and thus in the number of nodule isolates we were likely to obtain, we chose instead to collect soil samples and to isolate from these the rhizobia we needed for further study. This was done by inoculating samples of prairie soil onto surface-sterilized and pregerminated seedlings of the legumes for which we hoped to obtain inoculant-quality rhizobia. We then collected the nodules that resulted with each host, and isolated pure cultures of rhizobia from them. These growth and nodulation tests were undertaken using initially sterile

Leonard jar assemblies (23) each prepared from three Magenta units (Sigma, St Louis, Mo) as shown in Figure 3.1. In this, the top unit protects the seedlings from contamination, but allows air movement and photosynthesis; the central unit contains a support medium (silica sand/Sunshine mix No 2 (J.R. Johnson, Minneapolis, MN) 4: 1) on which the plant grows, and the bottom unit is part-filled with a low N plant nutrient solution (modified from Summerfield et al (33) and Smith et al. (34)). Piping filler cord (Wrights, West Warren, MA) connecting the central and lower chambers allows wicking of nutrient solution to the soil chamber as needed by the growing plants. Units were assembled, then sterilized by autoclaving at 1.38 x 10^5 Pascal for 20 minutes.

Figure 3.1: A Leonard jar assembly such as was used in trapping rhizobia from soil, and in the evaluation of strain effectiveness in nodulation and nitrogen fixation.

Seeds of the trap hosts *Amorpha canescens, Astagalus canadensis, , Chamaecrista fasciculata, Dalea purpurea, Desmodium canadense* and *Lespedeza capitata* (Prairie Moon Nursery, Winona, MN) were surface sterilized with 3% bleach or conc. sulfuric acid, depending on hard seededness (23), rinsed repeatedly in sterile distilled water, then pregerminated in covered pyrex dishes containing sterile, moistened sand. Germinated seed was aseptically transplanted into the magenta units 3-4 days after sterilization, and the plants inoculated with 5 ml of a 10^{-2} dilution of prairie soil. Plants were grown for 5-8 weeks in a Conviron PGW36 growth chamber (Controlled Environment Ltd., Winnipeg, Canada) at 25°C/20°C day night temperature and 14-hour photoperiod, then harvested, and the nodules each had produced were collected and washed. Rhizobia were isolated from these nodules as described by Vincent (35) with 50 to 150 rhizobial isolations made for each legume of interest, as shown in Table 3.2. These rhizobial isolates were then evaluated for nodulation and nitrogen fixation under growth chamber, glasshouse and field conditions with strains selected that were superior in

nodulation (estimated by the number or the mass of nodules produced) and effectiveness in symbiosis (estimated by plant dry matter production in a system where the plant was dependent on symbiotic nitrogen fixation for growth). In 12 trials with different host legumes and strains, a highly significant positive correlation was always obtained between nodulation and plant dry matter production. This facilitated selection of the most effective rhizobial strains.

Strains used in this study belong to several genera and species of root nodule bacteria (18,36), and so for convenience are referred to here only as rhizobia. All isolates have been conserved in a glycerol/peptone/ sucrose medium, and stored at -70^{o}C (37), with working cultures maintained on yeast extract mannitol (YM) medium (35) and grown at 28^{o}C.

Prairie Area establishment and management

Separate prairie areas were established in the dryland area of the University of Minnesota Sandplain Research Station at Becker, MN in Spring, 1999, Fall 2000 and in both Spring and Fall, 2002. Soil at the station is a Hubbard Loamy sand (sandy, mixed frigid Entic Hapludoll). In 1999 and 2000 planting followed rye; in 2002 the area used had been in fallow in 2001. In each case soil was ploughed and packed, then grasses, other forbs and legumes separately broadcast and raked in. Seed used in the establishment of each prairie were purchased from Prairie Moon Nursery (Winona, MN), and corresponded to their short grass prairie-dry mesic GM-025 seed mix. This varied slightly in composition from year to year depending on seed availability, as shown in Table 3.1.

Table 3.1. Composition of the seed mix used in each year of planting

Species	Common Name	% by weight in		
		1999	2000	2002
Forbs				
Agastache foeniculum	Anise hyssop	0.33	0.35	0.33
Asclepias tuberosa	Butterfly weed	3.00	3.17	3.00
Asclepias verticellata	Whorled milkweed	0.67	0	0.67
Aster azureus	Sky Blue Aster	1.00	1.06	1.00
Coreopsis palmata	Prairie coreopsis	1.00	1.06	1.00
Echinacea angustifolia	Narrow leaf coneflower	3.34	3.52	3.34
Eryngium yuccifolium	Rattlesnake master	3.00	3.17	3.00
Euphorbia corollata	Flowering spurge	2.00	2.11	2.00
Galium boreale	Northern bedstraw	1.00	1.06	1.00
Gentiana quinquefolia	Stiff Gentian	0.40	0.42	0.40
Helianthus occidentalis	Western sunflower	0.67	0.70	0.67
Liatris aspera	Button blazing star	2.00	2.11	2.00
Monarda fistulosa	Wild bergamot	0.67	0	0.67
Monarda punctata	Spotted bee balm	0.67	0.70	0.67
Penstemon grandiflorus	Large flowered beard tongue	1.33	0.47	1.33
Rabtibida pinnata	Yellow coneflower	0.67	0.23	0.67

Rudbeckia hirta	Black-eyed susan	0.60	0.63	0.60
Tradescantia ohiensis	Ohio spiderwort	1.33	1.41	1.33
Verbena stricta	Hoary vervain	0.67	0.70	0.67
Zizia aptera	Heart leaf golden Alexanders	1.00	1.06	1.00
Ceanothus americanus	New Jersey Tea	1.00	1.06	1.00
Legumes				
Amorpha canescens	Lead plant	2.00	2.11	2.00
Amorpha nana	Fragrant false indigo	2.00	2.11	2.00
Astragalus canadensis	Canadian Milk Vetch	0.27	0.28	0.27
Baptisia leucantha	White wild indigo	2.00	2.00	2.00
Chamaecrista fasciculata	Partridge pea	13.34	14.09	13.34
Dalea candida	White prairie clover	2.00	2.11	2.00
Dalea purpurea	Purple prairie clover	2.00	0.70	2.00
Dalea villosum	Silky prairie clover	2.00	0.70	2.00
Desmodium canadense	Showy Tick Trefoil	1.00	1.06	1.00
Lespedeza capitata	Bush clover	2.00	2.11	2.00
Grasses, sedges and rushes				
Andropogon scoparius	Little blue stem	20.01	21.14	20.01
Bouteloua curtipendula	Side oats grama	14.01	14.80	14.01
Elymus canadensis	Canada wild rye	10.01	10.57	10.01
Koeleria cristata	June grass	1.00	1.06	1.00

For the planting in 1999 a single area 30m x 30m was seeded, with three replicate areas each 6.1m x 4.5m subsequently delineated within the larger area. A further six areas each 9.0 m x 15.25m were planted in 2000 with three of these areas inoculated with rhizobia, and three areas left uninoculated (see below). Because of the difference in inoculation treatment, 3m wide border areas were left around each plot, and seeded without inoculation to the lower cost GM024 seed mix. The total area seeded was 70m x 30.5m. Finally in 2002 six areas 5 m x 6m were planted, with three seeded in spring 2002 and the remainder in fall, the same year. Figure 3.2 provides an overview of the plot layout.

Because of concern for the overwintering of fall-applied inoculant, and the nodulation of slow-to-germinate legume seed in subsequent seasons, the rates of inoculant application used in the different plantings were all greater than would have been used in an agricultural setting. For inoculant preparation in 1999 and 2000, cultures of each of the inoculant strains were streaked onto YM agar plates and incubated for 7 days at 28^{o}C, then plates of each culture were used to inoculate separate broth cultures of the same medium. Following three days incubation with agitation at 25^{o}C, each broth culture was separately mixed with granular peat (Nitragin, Milwaukee, WI) and calcium carbonate (ratio peat to calcium carbonate 11:1) to 28% moisture content, and allowed to stand overnight, then bagged in Ziploc storage bags, and maintained at 10^{o}C until used.

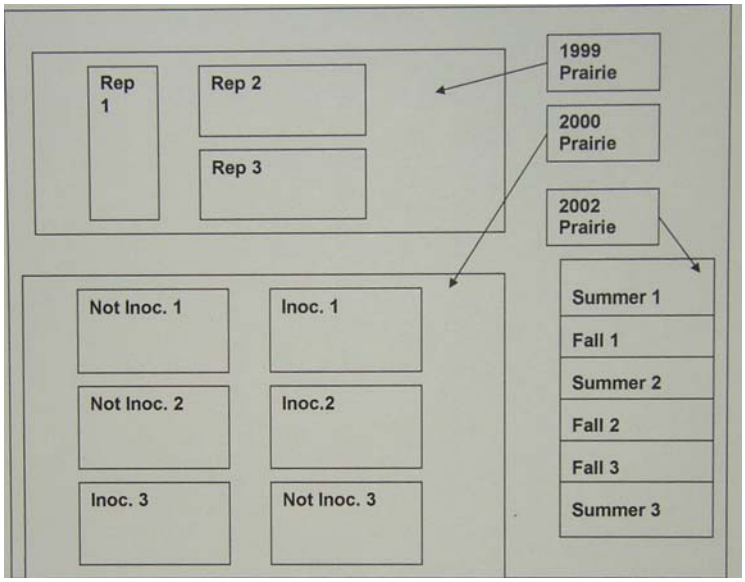

Figure 3.2. Overall layout of the prairie area at Becker showing the three separate prairie plantings, and replication within each

For field inoculation, the individual peat inoculants were mixed to provide approximately 5 x 10^7 of each rhizobial strain g^{-1} then broadcast over the field plots at a rate of 10 kg ha^{-1}, and raked in. For the 2002 planting the inoculants were prepared with the samestrains but using a clay-based proprietary formulation (MicroBioRhizogen, Saskatoon, Canada), then were mixed and incorporated as before. To further ensure nodulation, the area seeded in 1999 was reinoculated in spring 2000, while the inoculated areas seeded in 2000 were divided in two in spring 2001 and half the original area reinoculated. Entry to the uninoculated plots was controlled for the period of the study, with sterile protective footwear and gloves always worn during sampling.

To induce nitrogen deficiency in the prairie areas, and favor legume development, prairie areas were mown each fall, and plant residues raked off. Other management of the establishing prairies was limited to manual removal of invasive species, particularly *Vicia spp.* Uninoculated plots were always treated before those that had been inoculated.

Plant growth and development

Species composition, including that of invasive plants was determined for each prairie area in July of each year following prairie establishment, with particular attention paid to each prairie legume in the initial seed mix. Replicate plot areas were divided into 1m wide strips with species identifications made by two separate individuals. To avoid damage to the establishing prairies, and perhaps the eradication of plant species that were infrequent in occurrence in the first two years, samples to assess plant productivity and nitrogen accumulation were only taken in fall 2003. For these, 1 m^2 grids were placed at random in each plot, and the vegetation clipped and removed. Plant material was divided into legumes, grasses and other forbs, and then dried and weighed. Sub-samples were then ground and subject to total nitrogen analysis using the Dumas method (38, 39).

Soil chemical analysis

Soil samples from each prairie area were again only taken in July, 2003. Five soil cores each 25cm x 2cm were collected from randomly assigned locations in each replicate plot, and from the immediately adjacent field area, the samples for each replicate mixed and sub-samples taken for routine chemical and physical analysis at the University of Minnesota Research Analytical Laboratory. Procedures used in these analyses are detailed on the Research Analytical Laboratory website at http:ral.coafes.umn.edu/soil.htm. In addition, soils samples were analyzed for wet aggregate stability using a nested wet sieving technique (40), and for microbial biomass carbon, microbial biomass nitrogen and microbial respiration using the method of Jenkinson and Powlson (41) as described by Parkinson and Paul (42). These additional soil parameters were chosen as representative of those commonly used in the evaluation of changes in soil quality (43, 44, 45, 46).

Inoculant strain survival and contribution to nodulation

To estimate survival of the rhizobia needed for each legume over time in the soil of the different prairie areas, the soils collected for chemical analysis were also used to trap rhizobia, and to determine the relationship between these isolates and the initial inoculant strains. Trapping of the rhizobia used the Leonard jar system described above, with sterile pregerminated seeds of each prairie legume species transplanted aseptically into 72 Leonard jar assemblies, and four units each inoculated with 5 mL of a 10^{-2} dilution of soil collected from one of the18 different prairie areas (sowing in 1999, 2000 and 2002; not inoculated inoculated and 2 x inoculated areas in 2000; spring and fall sowing in 2002; three replicates of each). *Dalea purpurea* was used as trap host for both *D. purpurea* and *D. candida*; *Chamaecrista fasciculata* was not used as a trap host a) because of difficulties with seed germination and b) because both *Desmodium canadense* and *Lespedeza capitata* are also members of the cowpea cross-inoculation group and might be expected to recover the same organisms Plants were grown in Conviron PGW36 growth chambers for six weeks at $25^{o}C/20^{o}C$ day night temperature, a 14 hour photoperiod and 97% relative humidity, then were harvested and scored for the presence or absence of nodulation.

When a number of the plants in the initial nodulation assays failed to nodulate, whereas $\delta\ ^{15}N$ values showed that legumes in most of the plots were actively fixing nitrogen, we concluded that rhizobia might well be concentrated in the soil beneath their specific hosts. To test this with plant species for which *Rhizobium* recovery from random soil samples was variable, we collected soil from immediately below randomly selected *Dalea* and *Astragalus* plants in the 1999 and twice inoculated 2000 prairies. Each sample was then tested as indicated above.

Rhizobia in the nodules collected as part of the two different nodulation assays were then fingerprinted using Box A1R-PCR (47, 48) with up to 25 nodules (depending on availability) assayed per plant host per replicate. For PCR, washed and surface-sterilized nodules were placed in separate 1.5 mL microtubes on ice, 400 µL TE buffer (10:1, pH 7.6, 49) added, and nodules disrupted using sterile polypropylene micropestles. Nodule debris was removed by centrifugation at 1000 rpm for 15 min, then the supernatant transferred to fresh, sterile 1.5 mL microfuge tubes, and the bacteroids collected by centrifugation at 14000 rpm for 5 min. Bacteroid preparations were washed

in sterile TE buffer and recentrifuged, then resuspended in 50 μL TE, and stored at -20° C. BOX A1R-PCR used primer C-09 (CTA CGG CAA GGC GAC GCT GAC G) synthesized by IDT (Coralville, IA) and a PTC-200 Thermocycler (MJ Research, Waltham, MA). Ten μL aliquots of PCR product were then separated on 20 x 25 cm gels containing 1.5% agarose, with electrophoresis carried out at 70V for 17.5h. Each gel included the reference strain *R.etli* UMR1632 and three 1 Kb DNA ladders.Gels were stained with ethidium bromide and photographed using a Photodyne photo analysis system (Fotodyne Inc., Hartland, WI). Gel images were subject to quantitative analysis using Bionumerics version 3.0 (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 densiometric curve for each strain was analyzed using Pearson's correlation coefficient as a measure of similarity, then cluster analysis (UPGMA unweighted pair group method with arithmetic averages) used to generate dendrograms showing the phylogentic relationship among strains. Dendrogram data was then used to determine the percentage of nodule isolates for each host legume showing identity with the different inoculant strains used.

$\Delta\ ^{15}N$ nitrogen analysis

Because variation in the germination and emergence of individual plants, and the deep-rootedness of most legumes made it difficult to recover many of the nodules produced and to compare rates of N accumulation, we used $\delta\ ^{15}N$ analysis to estimate the % of N in each host derived from N_2 fixation (%NDFF). Samples for $\delta\ ^{15}N$ used the entire shoot of each plant to reduce partitioning error (22), and were taken from the 1999 prairie in July of 2002, and from all prairie areas in July, 2003. Where sufficient plants were available, five representative plants of each prairie legume species were harvested per prairie area. These were dried and finely ground, then analyzed for %N and $\delta\ ^{15}N$ using a 20-20 stable isotope mass spectrometer, with Pale yellow coneflower (*Ratibida pinnata*) as the non-fixing control. %N derived from fixation was calculated from the equation

$$\%N \text{ derived from fixation} = \frac{[\delta^{15}\text{N reference plant} - \delta^{15}\text{N legume}] \times 100}{[\delta^{15}\text{N reference plant} - B]}$$

where B is a measure of isotopic fractionation in a plant dependent on fixed N_2, and the reference plant has no detectable N_2 fixation. To determine B values for the plants used in this study, each of the legumes in question was grown without N supplementation in sterile sand, and fertilized with Fahraeus –N plant nutrient solution (23). They were inoculated with the appropriate rhizobia, and were dependent on N_2 fixation for growth. A B value of -1.86 was used in the above calculation, as being the average for the legumes tested.

Results and Discussion

Strain isolation and initial evaluation

Sources for the rhizobia which were evaluated for nodulation and nitrogen fixation with their respective hosts are shown in Table 3.2. As indicated in this Table 650 isolates were collected. Not all of these have yet been evaluated for nodulation and nitrogen fixation ability, a significant number of the isolates from *Dalea* have also been analyzed for differences in genetic diversity (see chapter 7).

Table 3.2. Sources for the rhizobia for prairie legumes used in this study

Prairie Sampled	*Amorpha canescens*	*Astragalus canadensis*	*Chamaecrista fasciculata*	*Dalea purpurea*	*Desmodium canadense*	*Lespedeza capitata*
Albert Lea State Park				3		
Anna Gronseth[1]	1	5				
Bluestem[1]	8	10	3	2		
Chippewa	14	8	7	9		
Hayden[3]	23				46	40
Helen Allison[2]	3				55	
Long Lake Park	63					
MNDOT rest stops				17		
New Ulm State Park		5		1		
Pankratz[2]	1	6	2	4		
Roscoe[1]	22	12		106		10
Springfield	1	1	6	6		
UMN-Arboretum	1	2		4		3
Warren		3				
Weaver Dunes[2]	6	7	39	6		
Windom	5	1	11	5		
Other	4		2	6	27	18
Total	152	60	70	224	73	71

[1] Department of Natural Resources Science Nature Area (DNR, 1995)
[2] Nature Conservancy site (50)
[3] Iowa

Twelve growth-chamber inoculation trials were undertaken in the selection of inoculant-quality strains for the six legume hosts. In each of these, and in subsequent greenhouse and field trials, a highly significant positive correlation between nodulation and plant dry matter production was obtained (Figure 3.3). This facilitated selection of the most effective rhizobial strains for use in the prairie study.

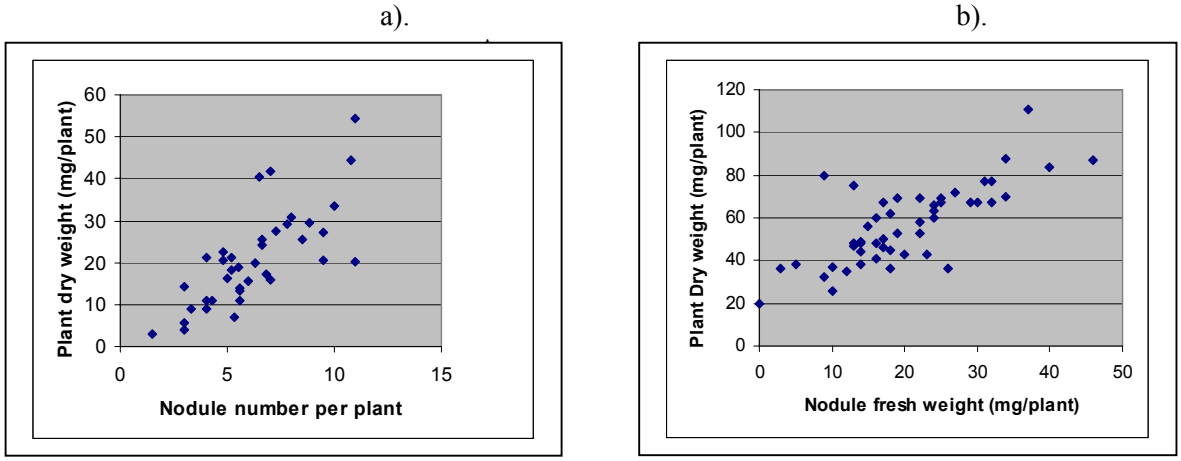

Figure 3.3: Strain selection for nodulation and nitrogen fixation in a) *Dalea purpurea* and b) *Lespedeza capitata.* In each case the strains selected for further study are those showing good nodule development and plant growth.

Table 3.3 identifies strains identified as being of inoculant quality, and used in the inoculation of the seeded prairies at Becker. The selected strains include representatives of the genera *Rhizobium, Bradyrhizobium* and *Mesorhizobium* (18, 36), but are referred to here only as rhizobia.

Table 3.3. Strains of rhizobia used in this study, and their origins

Strain	Inoculant strain for:	Source[1,2]
UMR 7520	*Amorpha canescens*	Long Lake State Park, MN
UMR7557	*Amorpha canescens*	Roscoe SNA, MN
UMR6335	*Astragalus canadensis*	Blue Stem SNA, MN
UMR6355	*Astragalus canadensis*	Pankratz Prairie, MN
UMR6404	*Chamaecrista fasciculata*	Weaver Dunes NC, MN
UMR6437[3]	*Chamaecrista fasciculata*	Weaver Dunes NC, MN
	Desmodium canadense	
UMR6808	*Dalea purpurea*	MNDOT Research Facility
UMR7205	*Dalea purpurea*	Helen Alison SNA, MN
UMR7240	*Dalea purpurea*	Roscoe SNA, MN
UMR6617	*Desmodium canadense*	CB3101, CSIRO, Australia
UMR6513	*Lespedeza capitata*	Hayden Prairie, Iowa
UMR6564	*Lespedeza capitata*	Hayden Prairie, Iowa

[1] Sites from which soils were collected, and nodule rhizobia isolated, are described in the Minnesota DNR (50) "A guide to Minnesota's scientific and natural areas"
[2] SNA and NC sources in this Table refer to Science Nature Areas and Nature Conservancy sites, respectively
[3] UMR6437 is an effective inoculant strain for both *Chamaecrista fasciculata* and *Desmodium canadense*

Prairie growth and establishment

Prairie plant establishment following seeding was slow and irregular, with each first-year "prairie" composed mainly of weeds from the seed bank. These included *Ambrosia artemisiifolia, Berteroa incana, Chenopodium album, Conyza canadensis, Crepis tectorum, Digitaria sanguinales, Krigia virginica, Lychnis alba, Polygonum convolvulus, Setaria viridis and S.glauca,* and *Thalspi arvense.* Of the seeded plants only *Monarda punctata and Rudbeckia hirta* were commonly identified in first year prairie seedings. Only isolated plants of *Chamaecrista fasciculata* were observed in the 1999 and 2000 plots one year after seeding, though 25-40 *Dalea purpurea, Dalea candida,* and *Chamaecrista fasciculata* plants were found in each spring-planted 2002 plot, in summer 2003. The combined effect of this initial pattern of establishment was a very high forb content in the first year. Incidence of weedy species declined dramatically in the second year with *Berteroa incana, Lychnis alba, Krigia virginica* and *Thalspi arvense* still most notable. At the same time, seeded non-legumes and grasses increased in frequency, with *Agastache foeniculum, Andropogon scoparius, Asclepias tuberosa, Bouteloua curtipendula, Echinacea angustifolia, Elymus canadensis, Helianthus doccidentalis,*

Koeleria cristata, Liatris spp., Monarda fistulosa, Penstemon grandiflorus, Ratibida pinnata, Rudbeckia hirta, and *Verbena stricta* most common. Legumes increased in frequency and diversity in this second year, but were still not frequent enough to sample. While the total number of legume plants recorded was not high (229 in the inoculated plots versus 135 in the uninoculated), seven of the eight legumes in the 2000 seeding, evaluated in the second year after planting, were more numerous in the inoculated than in the uninoculated prairie (data not shown). For the seeding made in 2000, *Chamaecrista fasciculata* was the most common legume species in 2002, but thereafter declined in frequency, with greater numbers of *Dalea candida, Desmodium canadense, Lespedeza capitata* and *D.purpurea* evident in both the 1999 and 2000 seedings, when evaluated in 2003. *Astragalus canadensis* and *Amorpha canescens* were slow to develop and in 2003 were still only common in the prairies planted in 1999. In the case of *Astragalus,* one reason for this could be the preference for it shown by transient deer. Repeated mowing of the 1999 prairie in mid-late summer has resulted in a well-balanced, grass and legume dominant prairie with few invasive plants. Change in the relative contribution of forbs, grasses and legumes to overall plant production of the prairie areas of different age, and the variation in total N (g m^{-2}) over time, is evident in Table 3.4. Notable in the latter is the difference between the inoculated and uninoculated prairies seeded in 2000.

Table 3.4. Grass, legume and forb production and nitrogen content in seeded prairies of different age, Becker MN, July 2003[1].

	Year prairie established					
	1999	2000 2 x I[2]	2000 1 x I	2000 NI	2002 Spring[3]	2002 Fall
Grass dry matter production (g m^{-2})	310	101	90	155	2	10
Grass % N	0.90	1.04	1.09	0.98	1.75	1.35
Grass total N (g m^{-2})	2.79	1.11	0.98	1.51	0.04	0.14
Legume dry matter production (g m^{-2})	7.73	0.76	0.10	0.56	1.03	0.17
Legume % N	2.34	1.94	1.40	1.91	2.60	2.28
Legume total N (g m^{-2})	0.18	0.02	<0.01	<0.01	0.03	<0.01
Other dry matter production (g m^{-2})	41	154	189	103	100	63
Other % N	1.20	1.37	1.01	1.03	1.42	1.17
Other total N (g m^{-2})	0.49	2.16	1.91	0.01	1.42	0.74
Total N (g m^{-2})	3.46	3.28	2.89	1.53	3.00	0.87

[1] Mean of triplicate samples

[2] Prairies established in 2000 were inoculated in 2000 and 2001 (2 x I), in 2000 alone (1 x I) or not inoculated (NI)

[3] Prairies established in 2002 were seeded in spring or fall, 2002

Soil chemical analysis

Soil properties for the seeded prairies developed during this project, and for the dryland farm soil from which they were derived, are shown in Table 3.5.

Table 3.5. Soil properties associated with seeded prairies of different age, Becker MN, July 2003[1]

Soil property	Dryland Soil	Year prairie established: 1999	2000 2 x I[2]	2000 1 x I	2000 NI	2002 Spring[3]	2002 Fall
Respiration rate[4]	ND[5]	10.1	5.8	3.4	3.1	4.7	3.6
Microbial Biomass C[6]	ND	114	134	141	165	141	126
Microbial Biomass N[7]	ND	16.3	12.2	11.3	10.0	12.8	9.9
Aggregate GMD	ND	0.61	0.63	0.61	0.58	0.64	0.53
pH	5.5	7.2	5.5	5.5	5.7	5.7	5.6
ppm Bray P	27	71	33	29	30	26	29
ppm K	81	131	132	118	110	78	84
% Organic Matter	1.3	2.20	2.03	1.93	1.76	2.26	2.13
Total N %	0.14	0.13	0.11	0.12	0.10	0.12	0.12
Total C %	0.81	1.18	1.12	1.10	0.95	1.13	1.09

[1] Mean of triplicate samples

[2] Prairies established in 2000 were inoculated in 2000 and 2001 (2 x I), in 2000 alone (1 x I), or not inoculated

[3] Prairies established in 2002 were seeded in either Spring or Fall, 2002

[4] $\mu g\ C\ g^{-1}\ day^{-1}$

[5] Not determined

[6] ppm C

[7] ppm N

Soils from the different prairie areas now exhibit a number of differences. Thus:
a) Respiration rate and microbial biomass N increased with time since prairie establishment, whereas microbial biomass C was lowest in the 1999 prairie. In the 2000 prairie, respiration rate and microbial biomass N were somewhat greater in the 2X and 1X inoculated areas, than in the area that was not inoculated. This effect is consistent with enhanced legume content, and with greater N_2 fixation in the older prairies.
b) In contrast, values for % organic matter, % N and % C, were all lower in the uninoculated prairie seeded in 2000. We attribute this to the effects of mowing and residue removal in the 1999 and 2000 prairies, balanced in the case of the older prairie by

increased organic matter production and N_2 fixation in 2003.
c) Soil from the prairie seeded in 1999 showed a dramatic increase in pH, P and (to a lesser degree) K content. This was not a reflection of fertilizer applications, which were uniform across prairies. We can only assume that it is the result of nutrient recapture from depth in the soil profile by the older, better established and possibly deeper-rooted plants of the 1999 prairie. M.J. Russelle and K. Schmidt (51) have shown rooting in both *Dalea purpurea* and Big Bluestem to below 1.5m over 14 months. Both van Noordwijk and Cadisch (52) and Kristensen and Thourp-Kristensen (53) suggest that deep-rooted plants may intercept nutrients otherwise lost to the system. We will continue to monitor these prairie areas, and if this hypothesis is correct, would expect to see similar changes in the younger prairies in the next 2-3 years.

Recovery of rhizobia and δ^{15} N analysis

Percentage of trap hosts nodulated following treatment with a 1:20 suspension of soil collected at random from the constructed prairies of different age varied with legume host and year of seeding. It was greatest for *Desmodium canadense,* least for *Astragalus canadensis* and *Amorpha canescens,* and was usually lower in the prairies seeded in 2000 than in those seeded in either 1999 or 2002 (Table 3.6). *Astragalus canadensis* was uneven in development in our prairies, and because it was preferentially grazed, rarely set seed. This meant some distance between plants, with rhizobial survival over two or more seasons in the absence of a host likely to have been very limited. The results for *Desmodium* are undoubtedly due to the ability of this legume to cross inoculate with a number of indigenous rhizobia (14, 15). Rhizobia were also recovered from a high percentage of the samples collected from beneath *Dalea* and *Astragalus* plants in the inoculated 1999 and 2000 prairies. We suggest that rhizobia from these perennial legumes might have difficulty surviving over time in the soil between plants, but can persist in the soil beneath their host. We hypothesize that in natural settings each perennial legume might then reinforce specific strains of rhizobia, and over time develop unique rhizosphere and nodule populations. This has implications for the effects of prairie fragmentation on *Rhizobium* population diversity in soil and should be studied further.

When BoxA1R-PCR was used to type rhizobia from random soil samples and from samples taken from directly beneath *Dalea* plants*,* a high percentage of the isolates exhibited genetic fingerprints similar to the inoculant strains UMR6808, UMR7205 and UMR7240, but few showed the banding patterns of UMR6815 (Table 3.6 and Figure 3.4). These two groups of organisms (UMR6808, UMR7205 and UMR7240 compared to UMR6815) cluster separately in PCR and 16S rRNA gene sequence analysis (see Chapter 7) with UMR6815 characterized by the ability to nodulate *Dalea purpurea* and *D. candida* but not *D.leporina.* Because of this we have decided to discontinue its use as a recommended inoculant strain. Though no inoculant strain from *Amorpha* was recovered from random soil samples, 59% of the rhizobia recovered from nodules using *Amorpha canescens* as trap host also showed identity with the three *Dalea* inoculant strains. This cross-inoculation between *Dalea* and *Amorpha* was not anticipated, but was also noted- in the reverse direction in the results reported in Chapter 7. Such nodulation promiscuity

Table 3.6. Recovery of rhizobia[1] from seeded prairies of different age, Becker MN, July 2003.

	Year prairie established					
	1999[3]	2000 2 x I[3]	2000 1 x I	2000 NI	2002 Spring[4]	2002 Fall
% trap plants nodulated (collected from random locations in each prairie):						
Astragalus canadensis	25	0	0	0	8	8
Amorpha canescens	66	0	17	0	0	0
Dalea purpurea[5]	66	25	8	8	83	66
Desmodium canadense	75	100	100	100	100	100
Lespedeza capitata	42	50	0	30	17	25
% trap plants nodulated (collected from beneath plants of the same species)						
Dalea purpurea	100	100				
Astragalus canadensis	62					
% recovery of inoculant rhizobia from random soil samples:						
Amorpha canescens (19)[6]	0[7]	-	0	-	-	-
Astragalus canadensis (0)	-	-	-	-	-	-
Dalea purpurea (80)	62	66	0	0	74	62
Desmodium canadense(129)	0	0	0	0	4	4
Lespedeza capitata (32)	0	0	-	0	0	0
b) From samples collected from beneath the indicated host						
Astragalus canadensis(67)	0	65	-	-	-	-
Dalea purpurea (37)	65	66	57	-	-	-

[1] For each sample 5 mL of a 1:100 dilution of soil was inoculated onto 4 surface sterilized and pregerminated seedlings of each legume. *C. fasciculata* was not included because it is nodulated by rhizobia from the cowpea miscellany (as are *Desmodium* and *Lespedeza*) and is very difficult to surface sterilize and pregerminate

[2] Five plants from each legume were used in the evaluation of %NDFF in 2003

[3] Prairies established in 2000 were inoculated in 2000 and 2001 (2 x I), in 2000 alone (1 x I), or not inoculated

[4] Prairies established in 2002 were seeded in either spring or fall, 2002

[5] Soil samples were collected randomly from each prairie, and in most cases did not coincide with any specific legume, or directly from beneath specific legumes

[6] Adequate numbers of nodules were not recovered from all treatments. Indicates the total number of nodules evaluated using BOX A1R-PCR for determination of nodule occupancy

[7] UMR7520, the inoculant strain used for *Amorpha* was not recovered from any nodule. However 59% of the rhizobia recovered from nodules of this host contained UMR6808. UMR7205 or UMR7240, the inoculant strains used for *Dalea.*

in species generally regarded as needing specific rhizobia for effective nodulation warrants further study. Rhizobia trapped from beneath *Astragalus* plants in the twice-inoculated 2000 prairie also showed the genetic fingerprint of the inoculant strain UMR6355; no isolate appeared similar to the second *Astragalus* inoculant strain UMR6335. While there was no evidence that indigenous rhizobia contributed significantly to the nodulation of *Dalea, Astragalus or Amorpha*, most of the rhizobia trapped using either *Desmodium canadense* or *Lespedeza capitata* as host did not correspond with any of the inoculant strains used. As indicated above, *Desmodium* species often nodulate with a range of indigenous organisms (14,15); *Lespedeza capitata* and *Chamaecrista fascilulata* can also show promiscuity in nodulation (12), though often to a lesser degree than for *Desmodium.*

Values for %NDFF varied from a low of 30% for *Astragalus* in the 1999 prairie/ 2002 sampling, to 100% for *Amorpha canescens* and *Dalea purpurea* in the 2000 prairie (Table 3.6). %NDFF was commonly in excess of 80%, explaining the build up of soil N in the older prairies. By the time samples were taken in 2003, there were no noticeable differences between the inoculated and non-inoculated prairie plots. All of the older prairie areas had well-established animal colonies, and showed substantial soil perturbation, and while we donned sterile boots before entering the uninoculated prairie areas, the animals did not.

This is the first study we are aware of to examine the inoculation and nitrogen fixation of an establishing prairie. Problems included uneven legume establishment and growth, the length of time inoculants were expected to remain viable before nodulation could occur, the greater predation of animals on some legumes than on others, and the effect of field animals in moving inoculant between inoculated and uninoculated areas. As suggested by Hargreaves et al (54) impossibly large areas or samplings would have been needed to ensure that the differences we recorded could be shown to be significant.

Figure 3.4. Recovery of isolates of rhizobia from soil samples taken from below plants of *Dalea purpurea* growing in the 1999 and 2000 2 X inoculated prairie areas. The figure shows the banding pattern for the strains recovered following BOXA1R-PCR, then clusters these on the basis of their similarity with the inoculant strains used for *Dalea.*

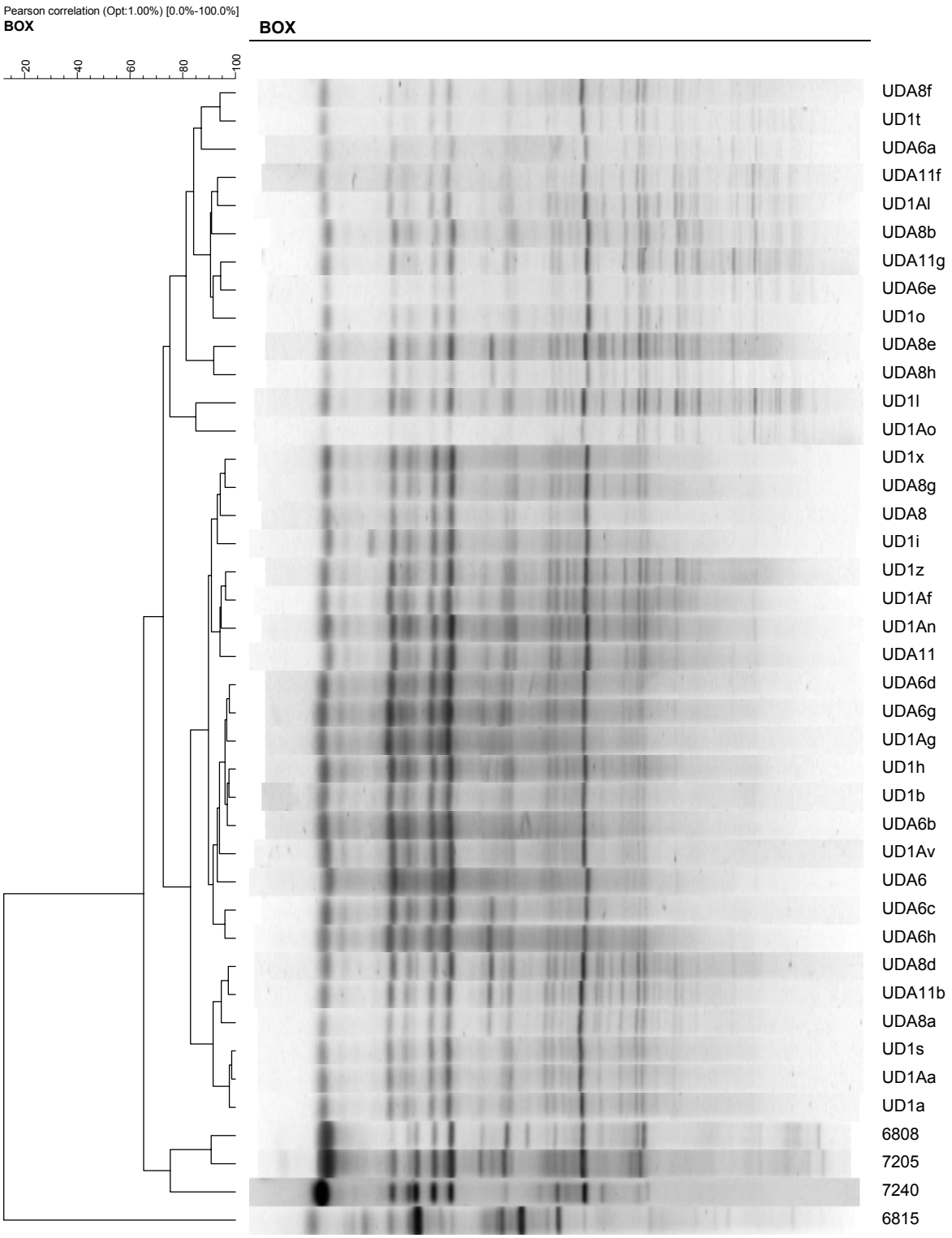
Pearson correlation (Opt:1.00%) [0.0%-100.0%]
BOX
20
40
60
80
100
BOX
UDA8f
UD1t
UDA6a
UDA11f
UD1Al
UDA8b
UDA11g
UDA6e
UD1o
UDA8e
UDA8h
UD1l
UD1Ao
UD1x
UDA8g
UDA8
UD1i
UD1z
UD1Af
UD1An
UDA11
UDA6d
UDA6g
UD1Ag
UD1h
UD1b
UDA6b
UD1Av
UDA6
UDA6c
UDA6h
UDA8d
UDA11b
UDA8a
UD1s
UD1Aa
UD1a
6808
7205
7240
6815

Table 3.7. Percentage of plant nitrogen derived from fixation (%NDFF) in legumes grown in establishing prairie areas of different age, Becker, MN., measured in July 2002 and July 2003[1].

	Year prairie established						
	1999[3]	2000 2 x I[2]	2000 1 x I	2000 NI	2002 Spring	2002 Fall	
	Measured in 2002[3]	Measured in 2003					
Astragalus canadensis	30	45	96	ND[4]	NF[8]	NF	NF
Amorpha canescens	94	82	100	ND	100	NF	NF
Chamaecrista fasciculata	NF	NF	97	ND	92	85	100
Dalea candida	68	77	78	ND	98	100	NF
Dalea purpurea	80	73	96	ND	100	75	NF
Desmodium canadense	85	73	68	ND	66	NF	NF
Lespedeza capitata	NF	96	97	ND	94	NF	NF

[1] Five plants from each legume were used in the evaluation of %NDFF using the procedure of Gathumbi et al. (22)

[2] Prairies established in 2000 were inoculated in 2000 and 2001 (2 x I), in 2000 alone (1 x I), or not inoculated. Prairies established in 2002 were seeded in either spring or fall, 2002

[3] In July 2002 the number of legumes evident in most prairies were limited and samples for δ ^{15}N analysis could only be collected from the prairie seeded in 1999

[4] Not determined

[5] The prairie indicated had insufficient plants of this species for sampling

At the same time we have provided some indicators of the importance of rhizobia, as well as legumes in prairie establishment, with the levels of prairie/legume dependence on biologically fixed N especially noteworthy. Brye et al. (55) suggest that a period of at least 5 years is needed to monitor prairie restoration progress. We agree, but also consider that significant information would have been lost if we had not taken these earlier samples. Thus, while there was considerable fluctuation according to prairie age in many of the traits we examined, the information collected will provide an excellent transition to subsequent studies. We have been advised that the University will maintain the prairie area at Becker (Figure 3.5) beyond the life of this project, and signpost the area as an attraction to visitors. This will afford us the opportunity for ongoing studies.

Figure 3.5. Overview of the area at Becker seeded in 1999 as it appeared in July, 2003.

Points that could be emphasized in subsequent evaluations with these prairies could include:

1. Brye et al. (55) note soil N accumulation in the top 30 cm of a 19-24 year old tallgrass prairie as 173 kg N ha^{-1} $year^{-1}$. In that study 77% of the N accumulated could not be accounted for and was presumably derived from N_2 fixation. This is a surprisingly high figure in a mature prairie, where buildup of soil N could be expected to lead to eventual inhibition of N_2 fixation (56) and must be a function of regular burning. While initial values for N_2 fixation in the prairies we have established are promising, we need to explore the influence of periodic burning on N accumulation in soil and how they influence rates of N_2 fixation. Carbon sequestration and soil quality changes should also be monitored.
2. In an agricultural situation it is possible to return after 10-15 years, and still to recover the original inoculant strain(s) from many of the nodules found (57, 58, 59). This is much less likely to happen in a situation where different legumes are present, and can interact with the rhizobia of another host, and where distances between legume plants can be significant. We will want to study strain persistence and change over time in these prairies.
3. The low numbers of rhizobia applied using conventional seed or soil inoculation, and the pronounced lag before their legume hosts are available for nodulation presents unique problems for the inoculation of prairie legumes. The use of montmorillonite clays with good survival features for *Rhizobium* in soil has been reported in Australia. The prairie might also be one environment where it is better to inoculate an alternate host, instead of

the legume(s). Diatloff (24) reported introduction of rhizobia for soybean via the inoculation of winter wheat, but such a practice in an agricultural crop rotation has rarely made sense. Its use with the cereal cover crops sometimes used in association with prairie plantings would allow uniform introduction of prairie rhizobia and permit high numbers of these organisms in soil in the first two years after seeding.

Recommendations

1. In earlier strain selection studies reported in our previous grant from MNDOT we showed that all of the legumes used by them in restoration activities, save perhaps *Amorpha canescens,* are active in nodulation and nitrogen fixation under controlled conditions. This project extended those studies to the field, and examined the importance of inoculation and of nitrogen fixation in the establishment and function of a tallgrass prairie. In this study both *Dalea* and *Astragalus* showed clear response to inoculation with the inoculant strains used for these hosts recovered from soil 2-3 years after application. *Lespedeza, Chamaecrista* and *Desmodium* appear more promiscuous in their nodulation behavior, with indigenous rhizobia from soil also contributing to their nodulation in the field. This problem seemed greater for *Desmodium* than for the other two legumes. Additional studies at other locations are needed to confirm these findings, and should attempt to include different inoculation strategies.. An unexpected finding in the present study (given that *Amorpha, Astragalus* and *Dalea*) are usually regarded as specific in their inoculant strain requirements) was that these mutualists appeared to interchange rhizobia. The implications of this, both for short term inoculation response and longer term strain persistence need to be further studied. We will continue to do this in the current prairie area, and in future prairie plantings.

2. Current inoculation methodologies for prairie seedings need to be improved. When inoculation occurs during the fall, it is unrealistic to expect that rhizobia should both survive through the winter and exist saprophytically in soil until they make contact with their host. At the very least this must compromise their ability to contribute to the nodulation of the more promiscuous hosts such as *Desmodium*. Even where granular inoculants are used, and applied at rates providing 10^{12} rhizobia per ha, the number of rhizobia recovered from the soil after the first year, and in the absence of an appropriate host, was not high. Alternate, and more effective methods of inoculation are needed. These might include reinoculation of the prairie in the second year, use of granular $Ca^{++/}Mg^{++}$-montmorillonite based inoculants which can be applied directly to the soil at seeding and which are suggested to protect the rhizobia from stress, or the seeding of a cover crop such as winter wheat or a prairie grass that could act as a surrogate host for rhizobia, and maintain high numbers of these organisms in the soil until legumes begin to establish. In a later chapter we report preliminary studies showing that winter wheat will maintain good populations of rhizobia for several weeks after inoculation. Because of the greater seed size of winter wheat (and therefore the number of rhizobia than could be attached to the seed, and because more seeds are planted per ha, much higher rates of inoculation could be achieved. Additional studies in these areas are needed.

3. Minor management was integral to the success of the prairie establishment at Becker. Legume establishment in whatever system requires that the area be driven toward nitrogen deficiency. In nature this is achieved by burning; in these prairies we mowed and raked the prairies to simulate burning. We also manually weeded the prairies to limit

early invasive species development. This is to some degree in conflict with current MNDOT recommendations where N fertilization rates are higher than is needed and could negatively affect prairie establishment and diversity

4. Nitrogen fixation by the prairie legumes used was impressive and appeared sufficient in only 3 years….even with mowing and straw removal…to effect significant improvement in soil properties. Longer-term studies should be undertaken to monitor effects on soil properties.

Chapter 4: Rhizobial inoculation, nodulation and nitrogen fixation of prairie legumes used in revegetation activities in Minnesota: Stillwater,MN.

Background

As part of this project we were also required to monitor prairie growth and development of a MNDOT wetland reconstruction site on Osgood Ave, Oak Park near Stillwater, MN. This site had been seeded prior to the award of our project in 2000, and our task was to separate out three areas to which inoculants were applied, and three areas where no inoculants were used, and then to monitor their development.

Methods

Methods used in this study were essentially as for the study at Becker.

Results and Discussion

Prairie growth and establishment

Plots at Stillwater were located on ground sloping toward the wetland area and so situated with inoculated and uninoculated areas placed side by side to avoid inoculant runoff into uninoculated treatments. As such they tended to be somewhat drier areas and better suited to growth of *Dalea spp* and *Chamaecrista fasciculata.* The area appeared to have been used previously in agriculture, and as such had numerous cultivated legumes present as "weeds" within the prairie area. Chief among these were *Melilotus alba, Trifolium pratense, Trifolium hybridum* and *Melilotus officinalis*. In 2000 and 2001 we saw evidence that attempts had been made to chemically treat these plants, but in 2002 and 2003 the area still contained significant populations of leguminous "weed species". This site was consistently more weedy than the Becker location. Weeds recovered from the plot areas sampled in 2001 and 2002 are shown in Table 4.1.

Table 4.1. Weed species recovered from inoculated and uninoculated plots in 2001 and 2002 at Stillwater.

Species .	Not Inoculated.		Inoculated	
	2001	2002	2001	2002
Melilotus alba White sweet clover			X	X
Ambrosia artemisifolia (ragweed)	X		X	X
Conyza canadensis (marestail	X		X	
Cirsium arvense (Canada thistle)	X	X	X	X
Urtica dioica (Stinging nettle)			X	
Plantago major (Common plantain)			X	
Solidago spp.		X	X	X
Eregeron annuus (Daisy fleabane)	X	X	X	X
Trifolium pratense (Red clover)	X	X		X
Melilotus officinalis (Yellow sweet clover)		X	X	X
Ambrosia trifida (Giant ragweed)			X	
Taraxacum officinale (Common dandelion)	X	X		X
Helianthus spp.	X			
Lactuca scariola (prickly lettuce)	X		X	

In contrast, the frequency of occurrence of seeded plants in our plots was low with only *Asclepias sp., Rudbeckia hirta (*Black-eyed susan) and *Monarda fistulosa* (Wild bergamot) among the forbs, and *Dalea candida* (White prairie clover) *, Dalea purpurea* (Purple prairie clover) *and Chamaecrista fasciculata* (Partridge pea)among the prairie legume species, routinely recovered. Presence of so many invasive legumes in our plot areas limited the meaning of any grass/forb/legume biomass measurement. In one of our plots *Melilotus alba* constituted 65% by weight of the plant biomass harvested. Distribution of the legumes, in particular, was very patchy with large clusters of *Dalea* and *Chamaecrista* on the western slopes around the wetland area, but limited frequency of them in other regions. This made sampling problematic.We found only one plant of *Desmodium* and no other prairie legume in these plots. Throughout the various studies made at Stillwater, it appeared that introduced plant diversity was very much reduced compared to that at Becker. This highlights the need for strong invasive plant and weed management in these areas.

Soil chemical analysis

Soil chemical analysis for random samples taken from triplicate plot areas at the Stillwater location in 2001, 2002 and 2003 are shown in Table 4.2.

Table 4.2. Soil properties associated with plot areas sampled at Stillwater from 2001 to 2003[1]

	Not inoculated			Inoculated		
	2001	2002	2003[2]	2001	2002	2003
pH[2]	7.3	7.3	6.5	7.6	7.6	6.8
Bray P (ppm)	50	50	38	58	58	37
K (ppm)	141	141	116	135	135	85
% Organic matter	2.6	2.6	2.5	3.5	3.5	2.8
% Nitrogen	0.14	0.14	0.14	0.19	0.19	0.14
Respiration rate (μg C g^{-1} day^{-1})	235			257		
Microbial biomass N (ppm N)	22			29		
Microbial Biomass C (ppm C)	316			373		

[1] Average for three replicate samples

[2] In 2003 soil samples for the inoculated treatments were taken from immediately adjacent to healthy *Chamaecrista* or *Dalea plants* and were averaged, those for the uninoculated treatments were taken from areas as far as possible removed from the influence of any legume.

There was significant variation in the soil results. We attribute this in part to the abundance of non-target legumes throughout the area, and perhaps to soil movement undertaken as part of the wetland reconstruction activities.

Recovery of rhizobia and δ ^{15}N analysis

We did not recover rhizobia for *Dalea* or *Astragalus* in soil samples collected from inoculated or uninoculated areas of the Stillwater prairie in 2002. For soil samples inoculated onto surface-sterilized *Amorpha* seedlings four of twelve plants inoculated

with soil from the inoculated area nodulated; none of those from the uninoculated plots did. For *Desmodium* and *Macroptilium* (used to trap cowpea-type rhizobia) % plants nodulated following inoculation with soil from the inoculated and uninoculated was in each case close to 50%, indicating significant numbers of these organisms in soil prior to our inoculation treatments.

Rhizobia were trapped from below *Dalea* plants in 2003, or from areas in which no such plants were evident, with frequency of noduled trap plants greater when soil was from below *Dalea* hosts than from areas with no *Dalea* plants. A total of 148 rhizobia were recovered, with 80% of these coming from below *Dalea.* For those recovered from beneath *Dalea* plants, 72% had genetic fingerprints similar to the three inoculant strains UMR6808, UMR7205 and UMR7240; none had the genetic fingerprint of UMR6815. This result is similar to that at Becker. For areas where no *Dalea* were evident 55% of the 68 strains tested also had the genetic fingerprint of our inoculant strains. The difference in % recovery of our inoculant strains in areas with and without *Dalea* is sufficient to suggest that our strains did take; their presence in areas with no *Dalea* enough to suggests that there was significant movement of seed and organisms on the sloping land around the wetland area. We have observed similar similar seed "erosion" at the MNDOT Cheri Ponds location. We attempted to undertake a similar analysis of the rhizobia for *Chamaecrista fasciculata,* but experienced consistently poor seed germination.

$\Delta\ ^{15}N$ values for *Dalea candida, D.purpurea* and *Chamaecrista fasciculata* plants harvested in 2003 were all between 95 and 100% NDFF. After three years it was obvious that these plants were flourishing.

Recommendations

It is difficult to the moment to make recommendations based on this site. This is in part because of the weedy nature of the site, but also because some of these "weeds" were legumes making it difficult to partition effects between introduced and invasive legume species. Soil perturbation in structuring the site also appears to have affected the variability of the data that could be obtained. While two of the legume species introduced are doing well and clearly benefiting from nitrogen fixation, the site as a whole does not have the diversity of plant species common to most prairie areas, while the legumes present seem clustered onto specific areas of the site. Further, because of the sloping nature of the site it was difficult to prevent movement of both seed and rhizobia into uninoculated areas. Management has to be a major factor in the establishment of high quality prairies and wetland sites, but it is probably the one most limited by cost and time issues. We are not familiar with methods needed to remove unwanted legume species, but what appears needed at Stillwater now that there has been opportunity for a seed bank of *Dalea* and *Chamaecrista* to establish, is for one or more wicking treatments with herbicide, coupled to mowing of the area prior to seed development, and the removal of plant material to enhance nitrogen deficiency and encourage further development of other prairie legumes and subsequently grasses. It appears to us that this area has not reached the same stage in its evolution as the prairie at Becker, and that concern has to be in the future diversity of plant species to establish there. Monitoring of this site is still needed, and perhaps the introduction/reintroduction of some of the currently scarce prairie species. Nitrogen fixation, in so far as *Dalea* and *Chamaecrista* are concerned does not appear to be an issue, reinoculation may be needed if other legumes are to be introduced.

Chapter 5: Rhizobial survival in prairie environments, and alternative strategies for greater rhizobial numbers and persistence

Background:

In the first two years of this project we undertook a number of studies attempting to identify better inoculant strategies for use in the prairie situation. We showed marked decline of rhizobial numbers in inoculants left at ambient temperatures in the field, with somewhat better survival of rhizobia applied as granular clay preparations at 10 kg ha $^{-1}$. (data not included).Even so, we continue to have concerns with the time inoculants must persist in soil prior to contact with their host. Because of this we welcomed the opportunity to work this summer with a high school student Thai Doan in preliminary studies aimed at determining if inoculation of cereal cover crops or prairie grasses might be an option for better strain persistence. As indicated earlier, several studies have suggested the possibility of inoculating cereals as a means to increase *Rhizobium* numbers in soil, and to improve strain persistence (57, 58, 59). Other studies even suggest that rhizobia can act as endophytic organisms in wheat, maize and rice, and as biostimulants, improving the growth of their surrogate host (60, 61). Mr Doan undertook two experiments in which winter wheats were inoculated and grown for 4 weeks in the growth chamber, then sacrificed and the number of rhizobia persisting determined.

Materials and methods

Eight winter wheat varieties were requested from Dr Joel Ransom, wheat specialist at North Dakota State University. We requested that the varieties cover differing periods of time since their release, and thus might differ in root exudates quantity and quality. The varieties obtained included Roughrider, Agassiz, Crimson, Harding, Jerry, Elkhorn, Ransom, and Seward.

Seed of each wheat variety was surface-sterilized using alcohol and hydrogen peroxide (35), then was pregerminated on sterile moistened sand and after two days planted into replicate magenta units, prepared and sterilized as previously described. Three seeds were initially planted per magenta, then thinned to one after 10 days growth. Inoculant for this experiment was a combination of the three current recommended rhizobia for *Dalea purpurea* UMR 6808, 7205 and 7240. Each was grown on BYMA medium as a check for purity then inoculated into BYMB and grown for 3 days on an incubator shaker at 24^{o}C. Counts of the cell density in each were then made using a Petroff-Hausser counting chamber, the density adjusted to 10^8 cells per mL, and the three cultures mixed. Cell density of the mixed inoculum was diluted to 10^5 cells ml^{-1}, with 5 mL inoculant applied to each magenta. Plants were grown for four weeks, then the young wheat plant carefully removed, suspended in dilution fluid and vortexed to shake off surviving rhizobia. The supernatant solution was then serially diluted 1:10^2 to 1:10^6, with aliquots plated onto yeast mannitol agar culture medium. These were grown for 3-4 days until colonies could be counted.

A second study used the same methodology, but only two of the initial seed sources. In this study each rhizobial strain was applied separately to identify possible strain differences.

Results and discussion

Results for the first trial with 8 different winter wheat varieties tested using a combined inoculant are shown below. All varieties supported at least 100 million rhizobia per seedling after four weeks growth, with significant differences between cultivars.

Table 5.1: Rhizobial survival in the rhizosphere of eight cultivars of seedling wheat, four weeks after planting

Roughrider	4.12×10^9 plant^{-1}	a
Agassiz	2.79×10^9 plant^{-1}	ab
Crimson	2.59×10^9 plant^{-1}	ab
Harding	3.06×10^9 plant^{-1}	ab
Jerry	2.60×10^9 plant^{-1}	ab
Elkhorn	1.54×10^9 plant^{-1}	ab
Ransom	8.16×10^8 plant^{-1}	b
Seward	4.28×10^9 plant^{-1}	a

In the second trial in which Roughrider and Seward were used as the varieties, and each tested using *Rhizobium spp.(Dalea)* UMR6808, UMR7205, and UMR 7240 as the inoculant strain, Roughrider and Seward with UMR6808 gave the highest rhizobial counts, with Seward with UMR7240 and UMR7205 significantly worse than the other treatments.

A poster prepared by Mr Doan, and based on this study has been selected as a semifinalist in the INTEL Science Talent Search

Recommendation

It appears from this initial study that inoculation of winter wheat applied as a cover crop could be a means to maintain higher levels of rhizobia in soil. Clearly however, much more experimentation needs to be done to study differences between varieties, strain effects, differences in compatibility between rhizobia in the rhizosphere, and persistence of both wheat and rhizobia over time. It would be desirable that a study be set up in which results with inoculation of the cover crop, or of the grasses present in the MNDOT seed mix be compared with those obtained using Ca- substituted montmorillonite clays as inoculant carrier.

Chapter 6: Inoculant production for prairie legumes

Background:

In 2003 the major producers of legume inoculants reduced the availability of inoculants for second-tier legumes such as those used in revegetation activities. Seed producers and workers in the revegetation field had to scramble for inoculants, and a number asked the *Rhizobium* Research Laboratory to make preparations for them. This we have done based on inoculant experience with other legumes, and without experimentation, but the activity has forced us to face a number of issues, best set down here. We would anticipate that inoculant production would continue into the foreseeable future.

Current inoculant capability and format:

As the result of experiments reported in this and other reports we have the expertise to produce inoculants using the following recommended strains:
Astragalus canadensis: UMR6355
Dalea purpurea and D.candida: UMR6808,UMR7205
Chamaecrista fasciculata: UMR6404, UMR6437
*Desmodium canadense:*UMR6617
Desmanthus illinoense: 36.10 or 56.6
Lespedeza capitata: UMR6564

We will prepare on request, but do not specifically recommend a culture for *Amorpha canescens* containing the strain UMR7520. After testing more than 100 *Amorpha* isolates we are still less than content with this strain.

For the preparation of inoculants, strains are first cultured onto agar plates to ensure purity and conformity to type. Growth from these is used to inoculate AG liquid medium (23), and this culture grown for 48 h at 24^{o}C with agitation, then 10 mL culture and 90 mL sterile water injected aseptically into a previously sterilized packet containing 125 g peat. The inoculated peat is manipulated thoroughly to ensure mixing.

We also currently undertaking experiments with three inoculant protectants (PBX-formerly Premax, Rizobacter, Argentina; PROTEC, Canada; Nitragin Optimizer protectant) which might be used in the application of either liquid or pre-sterilized peat inoculants with prairie legumes.

Issues

There are still questions to be answered about the inoculant cultures we have identified and their use. In testing in the Becker prairie it seems that the *Dalea, Astragalus, Lespedeza* and *Chamaecrista* inoculants are persisting in soil at minimal levels, but where we have tested it, do contribute to the nodulation of their host. Results with *Amorpha* and *Desmodium* are less satisfactory. At issue is how we can supply either more rhizobia, or better synchronize plant needs and microorganism supply. Results over a greater number of sites are also needed, and in particular must show that our inoculants persist in the soil over time. In the testing of inoculants for the major legumes trials will usually be undertaken with a number of groups working in different environments and with different cultivars. We are currently the only researchers in this field. Also at issue, as evident in our results is the cross-inoculation potential of rhizobia that have usually

been regarded as quite specific. How important is this in prairie development; how much is it affected by other plant species that are present? There are also questions of how others use inoculants in revegetation or nursery programs. We have seen packets subdivided and resealed, but no attempt made to ensure a water content that would protect the organisms; inoculants sold that contain a mix of prairie rhizobia; and inoculants stored open or for periods of time way beyond standard expiration times. We are currently developing an issue paper/manual describing in some detail methods appropriate to the storage and use of inoculants for prairie legumes.

Chapter 7: Differences in *Rhizobium* diversity and specificity according to seed and inoculant source: studies with *Dalea.*

Background

Habitat fragmentation is a common and serious environmental consequence of human expansion, often shaping ecosystems into areas that bear limited structural and functional relationship to the original (62). Such human activities have already transformed between 33 and 50% of the earth's land surface (63, 64), with consequences that include the extinction of some animal and plant species, and reduced population size, diversity and function in others (62). The tall grass prairie biome of the American mid-west is one area to have experienced this type of perturbation. In the early nineteenth century there were more than 7.29 million ha of tallgrass prairie in western and southern Minnesota; today less than 0.5% of that prairie area remains (1). Much of the residue is in areas inaccessible to farm machinery, along roads, rail lines and rights of way, and in various stages of degradation. Concern with this decline has prompted a number of active restoration programs, including the designation of specific highways as "Wildflower routes", but few restoration areas achieve their original condition (55, 65). A contributing factor could be the effects of fragmentation on symbiosis between prairie legumes and associated rhizobia. Legumes, are a major source of nitrogen in the tallgrass prairie (66), and prairie restorations in which legumes have failed to reestablish may be N limited and reduced in species richness even 25 years after seeding (67). Despite this, and the increasing interest in the relationship between above-and below-ground diversity (68), we know of only one study in which the below ground diversity of organisms in a tall grass prairie has been examined, and none that has considered the effects of diversity on *Rhizobium* diversity and function.

Fragmentation of the prairie areas has raised concerns of genetic erosion and loss, and has spurred efforts at reconstruction, with the Minnesota Department of Transportation one of several organizations participating in this effort. MNDOT is responsible for the maintenance of grassland areas alongside roadways, and has designated a number of these (for example highway 10 near Becker, MN) as "Wildflower Routes". Areas around rest stops on the major highways are also commonly planted to prairie plants, as a means of providing an attractive environment needing only limited attention post-establishment.

At issue in this program is the question of seed sourcing, with seed from local sources preferred. Where seed for specific projects is sourced in this manner it is likely that the productivity of restored areas will be affected both by the interaction of specific host and *Rhizobium* germplasm, and by the effects of fragmentation on the ecotypes used in seeding restoration areas.. In this study we have initiated research to explore the effects of seed source and fragmentation on *Rhizobium* diversity and function using the purple prairie clover *Dalea purpurea* as an example.

Materials and methods

Cone and soil sampling

In the fall of 1999 *Dalea purpurea* seed cones and soil samples were harvested from three prairies;

- Kellogg-Weaver Dunes, TNC, Wabasha County, MN;
- Ordway Prairie, TNC, Pope County MN, and
- Hayden Prairie, Howard County, IA.

The samples were collected with permission of the Nature Conservancy and the Minnesota and Iowa Departments of Natural Resources, but we were limited in seed sampling to only twenty plants and to collecting three 100g soil samples per prairie. The cones were selected from twenty plants > 3 meters apart and scattered throughout each prairie. The soil samples were taken to a depth of 10 cm, in close proximity to *Dalea* spp. populations, then stored at 5°C.

Effectiveness Assay

Cones from the Ordway prairie yielded too few mature seeds to be useful in further studies. Each cone from the Hayden and Kellogg/Weaver Dunes prairies was kept separate. Seeds from each cone were dehulled, scarified and stored at -20°C. To study their interaction with *Rhizobium* seeds were surface sterilized by rinsing 30 seconds in ethyl alcohol, followed by immersion for 4 minutes in 3% chlorox (35), then washed 5 times with sterile distilled water, and imbibed in sterile distilled water for 4 hours. They were pregerminated in sterile petri dishes containing silica sand for 2 days at 28°C. Seed germination percentage varied very substantially between cones. Fifteen cones from the Hayden and Kellogg/Weaver Dunes prairies, each having at least 9 germinating plants were randomly selected to be included in this study.

Plants were grown in coupled magenta units as described in Chapter 3. Variations in the technique used were that the lower unit contained 300 g of 3:1 industrial quartz #10 (Unimin, La Sueur, MN) and unfertilized peat moss (Sungro Horticulture Inc., Bellevue, WA) while the plant nutrient solution used was that of McDermott and Graham (69).

The three soil samples from each prairie were combined and mixed thoroughly. For each prairie,160 grams of soil was shaken in 3200 mL of sterile distilled water, with three replicate magentas of each of the 30 seed sources then inoculated with 100mL of the appropriate prairie soil. The magentas were arranged in a completely random pattern in a Conviron PGW36 growth chamber (Controlled Environment Ltd., Winnipeg, Man., Canada) with a 12 hour photoperiod and a 25°C day temperature and 20°C night temperature. They were thinned to one plant per magenta after 20 days, and harvested 107 days after inoculation. Nodule number and fresh weight per plant were determined, then nodules stored at -20°C for phylogenetic characterization. Plant shoots were separated and dried then plant dry weight determined.

Most probable number (MPN) count

Soil most-probable-number (MPN) counts (23) were used to estimate the *Rhizobium* population in each prairie. *Dalea leporina* var Sundance (70) was used as host, and seeds sterilized and pregerminated as indicated above. Two-day-old seedlings were then aseptically transferred into 25mm x 200mm culture tubes (Bellco Glass, Inc, Vineland,

New Jersey) containing 50 mL Summerfield-N plant nutrient solution (69) with 0.75% agar. For each MPN count, 10 grams of each prairie soil was diluted in 90 mL phosphate-buffered saline (49) and agitated on a wrist-action shaker for 10 minutes. A series of four-fold dilutions covering the range 1in 10 to 1 in 10,240 was prepared, with four replicate plants per dilution each inoculated with 1 mL of soil dilution (35). Plants were grown in a Conviron PGW36 growth chamber (Controlled Environment Ltd., Winnepeg, Man., Canada) with a 12 hour photoperiod and a 25°C day temperature and 20°C night temperature, and were harvested 4 weeks after inoculation and scored for nodulation.

Phylogenetic characterization of rhizobia: Box A1R-PCR

To test the diversity of rhizobia in each prairie, rep-PCR with BOXA1R primer was conducted on 20-50 nodules from each seed source/ soil inoculant combination. Nodules were removed from the -20^{o}C freezer and imbibed for 1 hour in sterile distilled water before further treatment. They were sterilized by soaking in ethyl alcohol and chlorox (35) then rinsed five times in sterile distilled water. Each nodule was then crushed in sterile yeast-mannitol broth medium (YM, 35) and streaked onto plates of YM agar medium. Plates were incubated for 6 days at 28°C then isolated colonies selected and re-subcultured. Reference strains used for comparison in PCR (Table 7.1) were prepared according to the same protocol. The strains were stored on slants at 5°C and separately in sucrose/peptone solution at -70^{o}C.

Strains were prepared for PCR analysis using 1 ul from a single colony on 4-6 day old YM plates. This inoculum was added to 1 mL sterile TY medium using a sterile disposable inoculation loop and shaken at 280 rpm for 24 hours. Cultures were then centrifuged at 16,000 g for 2 minutes, the supernatant removed, and the cells washed twice with1 mL sterile 1 M NaCl. They were then re-centrifuged for 4 minutes at 16,000 g, the supernatant removed and the pellet resuspended in 0.5 mL sterile Tris-EDTA (TE) buffer (49) and stored at -20°C.

For PCR, whole cells samples were further diluted (5 µl:20 µl) in sterile distilled water in sterile 96-well trays (Sarstedt, Newton, NC) and stored at -20°C. BoxA1R-PCR was carried out using C-09 BOXA1R primer (47) and the procedure of Rademaker and de Bruijn (71). The reaction mixture contained 1.5 mM $MgCl_2$, 2.5 µl Fisher 10X buffer B, 15.62 µl Ultra Pure distilled water (GIBCO), 0.2 µl dNTP mix (1:1:1:1, 100mM dNTP set, Invitrogen corp.), 0.22 µg BOXA1R primer (Integrated DNA Technologies, Coralville, IA), 0.15 µl *taq* polymerase (5 U/ µl Fisher Scientific), and 2 µl of whole cell suspension. Each set of reactions included a negative control with sterile distilled water substituted for the whole cell suspension. PCR was undertaken using a PTC 200 thermocycler (MJ Research, Waltham, MA) and the following protocol: incubation time was 4 minutes at 94°C, 30 cycles of 94°C for 3 seconds, 92°C for 30 seconds, 53°C for 1 minute and 65°C for 8 minutes and a final termination step at 65°C for 8 minutes. PCR product was stored sealed at -20°C until used.

Five µl 6X loading dye (49) was added to each PCR product, then 10 µL of this solution loaded into one well of a 20 x 25 cm horizontal electrophoresis gels containing 1.5% agarose. Solutions of a 1kb DNA ladder (Promega Corp.) were included on the outside lanes and in a middle lane of each gel, and the PCR product of the strains *Rhizobium spp* UMR6815 and *Rhizobium etli* UMR1632 included on each gel as

standards. Gels were run under a constant temperature of 8°C at 70V for 17.5 hours. They were then stained in 0.5 μg/ml ethidium bromide for 30 minutes, and photographed using a FOTO/Analyst Archiver (Fotodyne Inc., Hartland, WI). Gel images were subject to quantitative analysis using Bionumerics version 3.0 (Applied Maths,

Table 7.1. List of reference and known strains used in this study

Species	Strain (Synonym)	Host of Isolation	Source
Bradyrhizobium spp. (Chamaecrista)	UMR 6404	*C.fasciculata*	1
Mesorhizobium huakuii	UMR6355	*Astragalus canadensis*	1
M. huakuii	UMN6931 (USDA3135)	*Astragalus sinicus*	3
M.huakuii	UMN6932 (USDA3348)	*Astragalus alpinus*	3
M.amorphae	UMR7520	*Amorpha canescens*	1
Rhizobium etli	UMN1632 (CIAT 632)	*Phaseolus vulgaris*	1
R.gallicum	UMN 6918 (R602)	*Phaseolus vulgaris*	2
R.gallicum	UMN6922 (PhF 29)	*Phaseolus vulgaris*	2
R.giardini	UMN6917 (H152)	*Phaseolus vulgaris*	2
R.leguminosarum	S 34	*Phaseolus vulgaris*	4
R.leguminosarum	S 20	*Phaseolus vulgaris*	4
R.tropici IIA	UMN 6915	*Phaseolus vulgaris*	1
*R.tropici*IIB	UMN1899 (CIAT 899)	*Phaseolus vulgaris*	1
Rhizobium spp.(Coronilla)	UMN6874	*C.varia*	1
Rhizobium spp.(Dalea)	UMN6808	*Dalea purpurea*	1, 5
Rhizobium spp.(Dalea)	UMN6815	*Dalea purpurea*	1, 5
Rhizobium spp(Dalea)	UMN7205	*Dalea purpurea*	1, 5
Rhizobium spp (Dalea)	UMN7240	*Dalea purpurea*	1, 5
Rhizobium spp. (Onobrychis)	UMN 6861 (116A12)	*On. viciifolia*	1, 6
Rhizobium spp.(Onobrychis)	UMN 6871	*On. viciifolia*	1
Rhizobium spp.(Oxytropis)	UMN 6929(USDA3119)	*Ox.riparia*	3
Rhizobium spp.(Oxytropis)	UMN6930(USDA3121)	*Ox.riparia*	3

1. University of Minnesota *Rhizobium* Research Laboratory
2. Noelle Amarger, INRA, France
3. USDA Culture Collection, Beltsville
4. G.R. Bernal (72)
5. Tlusty et al. (73)
6. Courtesy R.S.Smith (Nitragin)

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 used as a measure of similarity. Cluster analysis used the UPGMA unweighted pair group method with arithmetic averages to generate dendrograms showing the phylogenetic relationship among strains. From the dendrogram, the diversity of the rhizobia population in each prairie was calculated using the Shannon diversity index (74):

$H' = -\sum p_i \ln p_i$

Where p_i is the proportion of rhizobia strains found in each group of strains at > 75% similarity divided by the total number of strains found in each prairie soil.

Phylogenetic characterization of rhizobia: 16S-rRNA sequence analysis

16S rRNA gene sequence analysis was carried out with fourteen strains representative of clusters separated using BOXA1R-PCR plus the *Dalea* inoculant strains UMR6815, UMR7205 and UMR7240, and used the methods of van Berkum et al. (75) and the primers 16Sa and 16Sb of van Berkum and Furhmann (76). The 16S rRNA genes were amplified in 120 µL volumes, and PCR products purified using QIA quick spin columns (Qiagen, Inc., Chatsworth, CA). Sequences were determined using a Perkin Elmer model 377 DNA automated sequencer in combination with a DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). Aligned sequences were analyzed as described by van Berkum and Fuhrmann (76).

Host-*Rhizobium* specificity

When 16S-rRNA sequence analysis identified strains with close similarity to *M.huakuii* and *M.amorphae* among the isolates recovered from *Dalea,* we decided to evaluate the host range of representative organisms identified in this study. Host-range testing utilized the magenta units and methods described in Chapter 3, with *Amorpha canescens, Astragalus canadensis, Coronilla varia, Dalea leporina* and *D.purpurea, Leucaena leucocephala, Macroptilium atropurpureum, Onobrychis viciifolia, Phaseolus vulgaris and Vicia americana* as host legumes, and the rhizobial isolates *M. huakuii* UMR6335, *M. amorphae* UMR7205, *R.etli* UMR1632, *R.gallicum* UMR6918, *R.leguminosarum* S20 and S34 (72), *R.tropici* UMR1899*;* and *Rhizobium spp (Dalea)* UMR6808, 6815, 7205, 7240, 1WW4, 1WW15, 3WH5, 4HR1, *Rhizobium spp (Coronilla)* UMR6874, *Rhizobium spp. (Onobrychis)* UMR6871 and *Rhizobium spp. (Oxytropis)* UMR 6929. Plants were inoculated with 5 mL of a 1 in 100 dilution of the appropriate culture, with two duplicate magentas of each host/strain combination tested, and grown for 4 weeks under standard growth chamber conditions before evaluation for nodulation and nitrogen fixation.

Results and discussion

Soil rhizobial populations in the three soils varied substantially with that of the Ordway site substantially less than found with either the Hayden or Kellogg-Weaver Dunes prairies (Table 7.2). In contrast to this, soil from the Ordway prairie showed greater diversity in the rhizobia it contained, and generated more and bigger nodules, and greater average plant dry weight with Hayden seed than did soils from either the Hayden or Kellogg-Weaver prairies. We could not explain this disparity until we completed PCR

and rRNA studies, and showed that most of the strains of rhizobia recovered from the Ordway site identified with strain UMR6815, recognized in the16S rRNA studies as belonging to the species *R.gallicum*. Tlusty et al. (73) had previously noted the ability of UMR6815 to effectively nodulate *Dalea purpurea* and *D. candida* but not *D. leporina,* the host used in MPN counts. This highlights once again, as earlier noted by Laguerre et al. (77) and Bernal et al. (78) the importance of host genotype in apparent differences in rhizobial population size and diversity in soil.

Table 7.2. Soil *Rhizobium* and nodulation and plant traits for *Dalea* rhizobia from the three different prairie soils

	Hayden soil	Ordway soil	Kellogg/Weaver Dunes soil
MPN count (rhizobia g^{-1})	2.50	<4.4	180
Shannon diversity index (H')	0.444 a[1]	2.221 c	1.361 b
Hayden seed source			
<>Mean nodule number pl^{-1}	3.33	4.57	2.93
<>Nodule weight pl^{-1} (mg)	12	16	15
<>Plant dry weight (mg)	20	22	18
Kellogg/Weaver Dunes seed source			
<>Mean nodule number pl^{-1}	3.07	2.29	2.20
<>Nodule weight pl^{-1}(mg)	11	13	10
<>Plant dry weight (mg)	14	13	12

[1] Data in a row not sharing a common letter are significantly different at 5% significance level

The dendrogram shown in Figure 7.1 compares BOXA1R banding patterns for the 211 strains of rhizobia trapped from soil collected from three prairie areas in Minnesota and Iowa, and using *Dalea perpurea* as host. It has been collapsed down for reproduction with the triangular areas each including multiple strains having greater than 75% similarity. Reference strains identified in Table 7.1 were included for comparison. Four major and 18 minor clusters of organism can be separated on the basis of banding pattern similarities, with each major cluster also including known isolates or reference strains (inoculant strains UMR7205 and UMR7240 from *Dalea* in Cluster 1; UMR 6931 from *Astragalus sinicus* in cluster 2, and inoculant strain UMR6815 from *Dalea* and UMR6861 from *Onobrychis* in cluster 3). However no representative of a recognized taxonomic species clustered in any major group.

There was also marked distinction between the isolates according to the prairie from which they were isolated. Thus 82 of 87 isolates in cluster 1 were from the Hayden prairie, all 59 isolates in cluster 2 were from Weaver Dunes, and 29 of 31 isolates in cluster 3 were from the Ordway prairie. We were only permitted to take three soil samples from each prairie, and so cannot extrapolate from this data to the entire prairie. However, either each sample taken showed marked spatial effects, or location and prairie history had a profound influence on strain diversity.

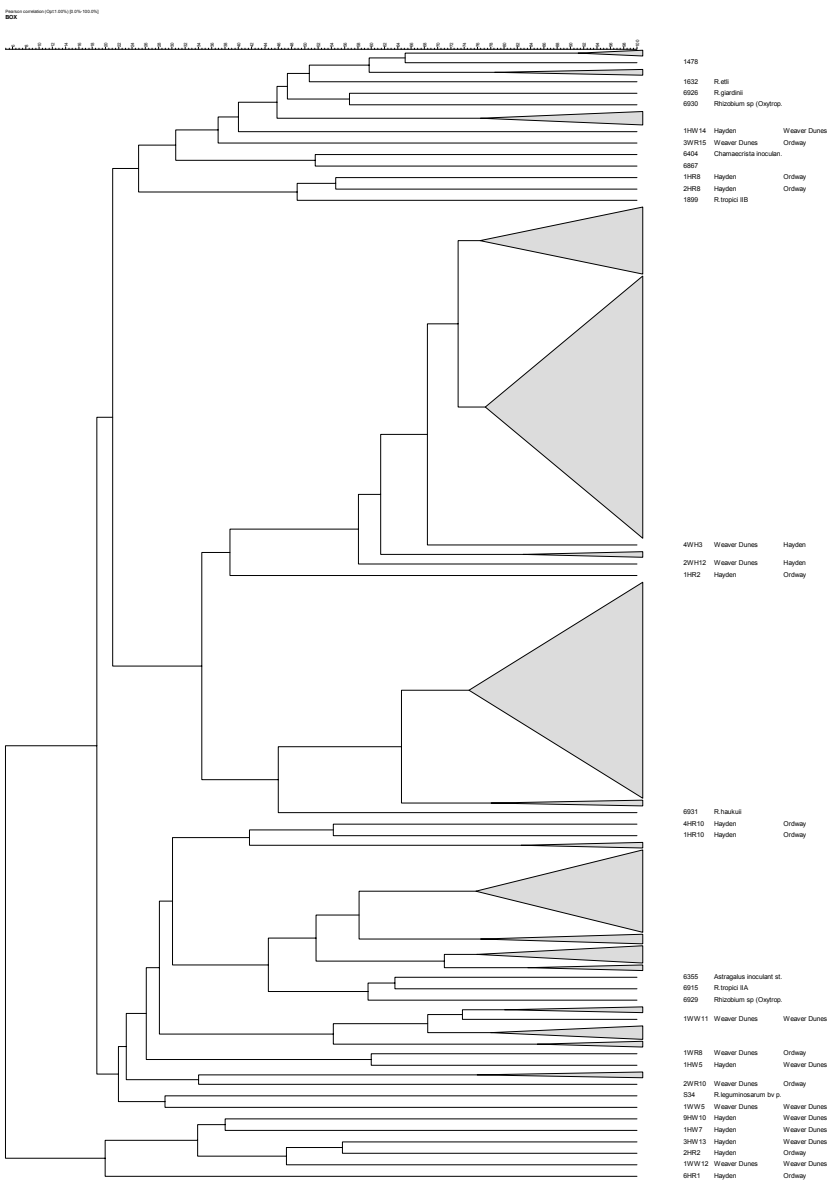

Figure 7.1. Dendrogram resulting from BoxA1R banding pattern analysis of 211 strains of rhizobia isolated from soils in the three prairies. The double triangular areas toward the top of the figure includes 86 isolates most from Hayden prairie and including UMR7205, UMR7240 and 3WH5; the next triangle area, 59 strains from Kellogg/Weaver Dunes including 1WW4; and the fourth large triangle, 32 strains most from the Ordway prairie. The minor cluster, third from the bottom includes 2HW3, 5HW3, 1WW15 and 1HW5. Nineteen strains representing different taxonomic species are included in the dendrogram, but failed to group closely with any of the major or minor clusters.

When these results are combined with those of the 16S rRNA gene sequence analysis (Figure 7.2) they are even more puzzling. According to this, all six strains taken as representative of the organisms included in cluster 1, including the two inoculant

strains UMR7205 and UMR7240, show greatest identity with *R. etli* and *R. leguminosarum.* Previous taxonomic studies have concluded that *R.etli* is relatively restricted in host range, though one biovar of this species is known to nodulate *Mimosa* (79). However, Graham et al. (18) previously pointed to similarity of some rhizobia from *Dalea* to *R.leguminosarum* and *R.etli*; while both Beyene et al (80) and Bernal et al. (72) have noted the difficulty in distinguishing *R. etli and R. leguminosarum.*

Four strains representative of those taken from the Ordway prairie, and that grouped as cluster 3 as the result of BOXA1R-PCR genetic fingerprinting were identified as allied to *R. gallicum.* Included in this group were the inoculant strain UMR6815 and a strain from *Onobrychis.* Since overlap between subsets of *Dalea, Onobrychis, Oxytrophis* and *Phaseolus vulgaris* microsymbionts have been suggested before (17, 18, 81), and similarity shown in fatty acid methyl ester (FAME) composition between rhizobial strains from *Dalea* and *R.gallicum*, this result is not unexpected. It does suggest however the need for more detailed studies with this group of organisms.

Two strains selected as representative of the 59 strains from Cluster 2 from Weaver Dunes and three isolates selected to represent 10 strains in a smaller cluster, all showed affinity to *M.huakuii* and *M.amorphae.* Rhizobia from *Astragalus* have been reported to nodulate *Onobrychis* and *Oxytropis* but in that report were not studied in relation to *Dalea* (17); Graham et al. (18) reported a single strain from *Dalea* to cluster with *M. huakuii* on the basis of FAME analysis. The number of strains involved in the current analysis, and the relationship with *M. amorphae* was, however unexpected. It should perhaps not have been. At almost the same time in a seeded prairie at Becker, MN, we trapped rhizobia from soil using *Amorpha canescens* as host, and showed that these strains had the genetic fingerprint of the *Dalea* rhizobia used as inoculant (see chapter 3). Wilson (82) has also noted nodulation between a strain from *Dalea alopecuroides* (syn. *D. leporina*) and *A. canescens, A. elata, A. fructicosa* and *A. microphylla*. As *Dalea* and *Amorpha* each fare best in somewhat drier environments, and are commonly associated, the frequency of interchange in their rhizobia is potentially of significance, especially in fragmented prairie areas. Weaver Dunes is a Nature Conservancy site with a previous history of at least partial use in agriculture. This could have affected persistence of *Dalea* rhizobia in the soil, and contributed to our current results. We are currently working with the rhizobia within fragmented prairie areas of western Minnesota which vary in the spatial distribution, and even the presence of *Dalea* and *Amorpha.* It will be interesting to see the effect of host representation and distribution on the species of rhizobia recovered.

Host range for some of the representative rhizobia from this study, and representative reference strains are shown in Table 7.3. Given the rustic nature of some of these hosts, it has been difficult to obtain adequate plant numbers for uniform results. Of interest in this data however is the broad promiscuity shown by *Phaseolus vulgaris, Macroptilium atropurpureum, Leucaena luecocephala, Onobrychis viciifolia* and *Dalea* species with various of the rhizobia tested, the nodulation of isolates 1WW15, 3WH5 and 1WW4 with *Astragalus canadense,* and nodulation of 1WW4 with *Amorpha canescens.* These results are consistent with those obtained using16S rRNA and BOXA1R-PCR.

Table 7.3. Host range for rhizobial strains possibly associated with *Dalea spp.*

Rhizobial strain	Host[1]										
	Am.c	As.c	Co.v	Da.l	Da.p	Le.l	Ma.a	On.v	Ox.l	Ph.v	V.am
UMR1632	----	----	----	----	----	----	-----	----		+++	----
UMR1899			+++		+++	+++	+++	+++		+++	----
UMR6355		+++								----	
UMR6808	+/-	----	+++	+++	+++	----	+++	+++		+++	----
UMR6815	----	----	+++	----	+++	+++	+++	+++		+++	----
UMR6871	+++	----	+++	+++	++++	----	+++	+++		----	----
UMR6874	+/-	+++	+++	----	----	----	----	+++		+++	----
UMR6918	----	----	+++	+++	+++	+++	+++	+++		+++	----
UMR6929	----	+++	----	----							
UMR7205	----	----	----	+++	+++	+++	+++	+++		+++	----
UMR7240	----	----	+++	+++	+++	+/-	+++	+++		+++	----
UMR7520	+++	----	----	----							
S20	----	----	----	----	----	----	----	----		+++	----
S34	----	----	----	----	----	----	----	----		+++	----
1WW15	----	+++	----	----		----	----			----	----
3WH5	+++	+++	+++	+++	+++	+++	+++			+++	----
4HR1	----	----	+/-	----	+++	+++	+++			+++	----
1WW4	+/-	+++	----	----	+++	----	+++			----	----

[1] Host plants used in this study were: *Amorpha canescens, Astragalus canadensis, Coronilla varia, Dalea leporina, Dalea purpurea, Leucaena leucocephala, Macroptilium atropurpureum, Onobrychis viciifolia, Oxytropis lambertii, Phaseolus vulgaris* and *Vicia americana.*

Recommendation

1). While it is outside the normal bounds of the projects funded by MNDOT, it is clearly important that the host range of the rhizobia associated with prairie legumes/ or used in revegetation activities, and how this might impact strain occurrence and diversity in fragmented prairies, be better defined. We recommend that MNDOT provide written support to projects that address this question.

2). In this study we were allowed only limited access to prairie samples, and thus it was difficult to distinguish between effects due to specific samples taken, and those due to the prairie evaluated. Broader studies are needed, that consider the effect of sample source, and the degree to which to which fragmentation is an issue, on the diversity of host and rhizobia likely to be available within locally available ecotypes.

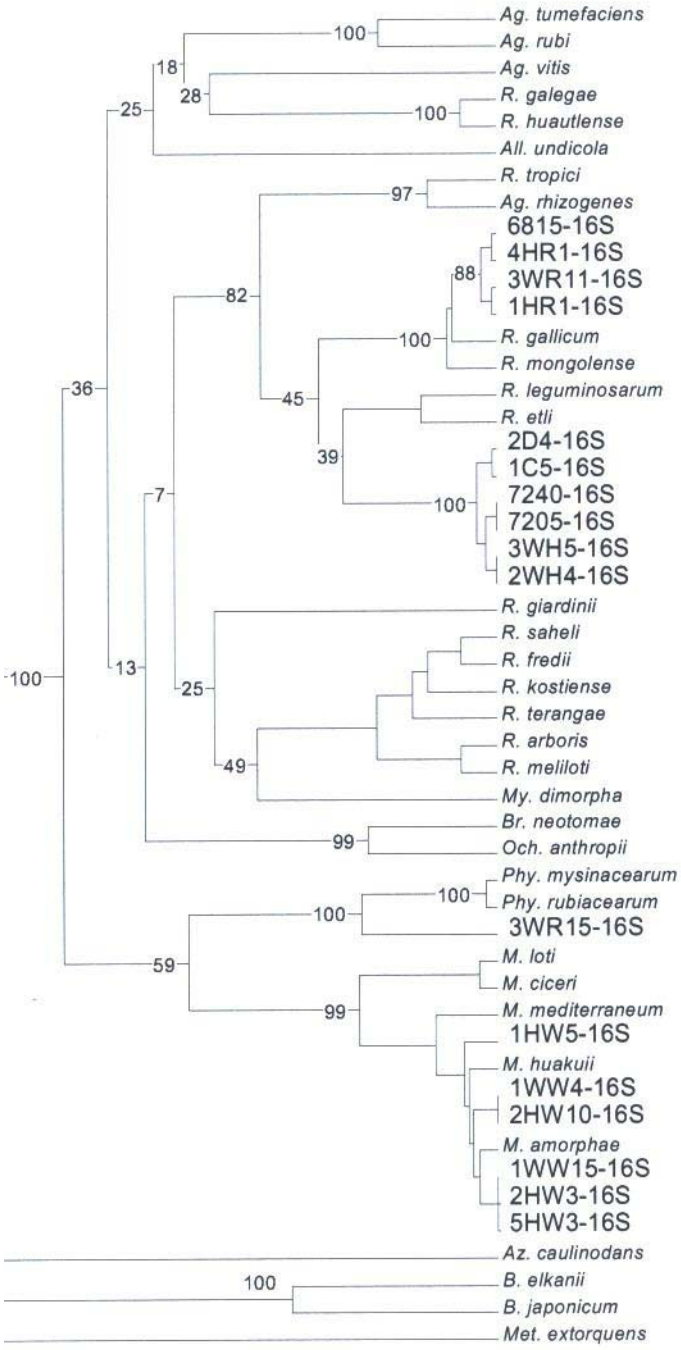

Figure 2. Clustering among 17 strains of *Dalea* rhizobia based on 16S rRNA gene sequence analysis. Each strain is representative of one of the clusters identified by Box A1R-PCR analysis of 211 strains from the Hayden, Ordway and Kellogg/Weaver Dunes prairies. UMR6815, UMR7205 and UMR7240 are inoculant-quality strains for *Dalea*. Sequences alignment used the PILEUP program in the Wisconsin package of the Genetics computer group, with sequence analysis using the Molecular Evolutionary Genetics Analysis (MEGA) software version 1.02. Levels of support for the presence of

nodes were obtained from 500 permutations of the dataset, and are indicated. (Data in this figure was obtained in collaboration with Peter van Berkum, USDA-ARS, Beltsville).

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