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**Properties of Symbiotic Nitrogen-Fixing Bacteria Isolated from
Common and Endangered Legumes**

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Summary

Much is known about the legume-rhizobial symbiotic relationship among crop plants such as soybeans. However, little information is available relative to the interaction of wild, native prairie legumes and their microbial symbionts or how this symbiosis impacts the competitiveness and survival of leguminous plants, both endangered and common species, in their native habitats. The goal of this project was to study nodulation and the microbial symbionts present in the nodules of endangered and common wild prairie plants in Illinois. In greenhouse studies, *Astragalus crassicaarpus* (an endangered plant species found at a gravel prairie at Beaver Dam State Park in Macoupin County, Illinois) developed root nodules when germinated seeds were inoculated with the prairie soil from Beaver Dam State Park. Microorganisms present in soil from Loxa Prairie (a mesic prairie in Coles County, Illinois) did not nodulate the roots of *Astragalus crassicaarpus* or the common prairie legumes *Amorpha canescens*, *Lespedeza virginica*, or *Petalostemum candidum*; root nodules were also not formed by *Astragalus crassicaarpus* or *Petalostemum candidum* when exposed to soil from Green River Conservation Area (a dry, sandy prairie in Lee County, Illinois). Likewise, microbes present in soils from Beaver Dam State Park did not nodulate the root systems of *Amorpha canescens*, and only 1 of 3 plants of *Petalostemum candidum* was nodulated when exposed to Beaver Dam State Park soil. These results indicate a high degree of specificity among the microbial symbionts present in prairie soils relative to host plant species. Microbes in nodules from *Astragalus crassicaarpus* were isolated on YEMA (a differential medium). Initial tests suggest that these isolates are members of the genus *Rhizobium* or *Bradyrhizobium*. Further studies to identify and characterize these isolates are presently underway.

Introduction

The legume-*Rhizobium* symbiosis is one of the most interesting and important plant-bacteria interactions in nature (1). Legumes are a diverse group that includes such economically important crop plants as soybeans and alfalfa and such native prairie plants as lead plant and prairie clover (2). Bacterial species in the genera *Rhizobium* or *Bradyrhizobium* (collectively referred to as rhizobia) are normal components of most soils and can infect the roots of leguminous plants. Infection of a given legume is highly specific, requiring a specific rhizobial species, and leads to the formation of root nodules that are

capable of nitrogen fixation. In nodules, bacterial symbiots provide the plant with reduced nitrogen in the form ammonia while receiving carbon and energy from the host plant (3). Thus, symbiotic nitrogen fixation by nodulated leguminous plants is a metabolic process that is vital to both agricultural and native terrestrial ecosystems. However, to date, most research has centered on the legume-*Rhizobium* symbiosis in crop plants and its overall influence on crop yields. Little, if any, information is available on the types of rhizobia that nodulate prairie legumes or the potential impact that these symbiotic nitrogen-fixing bacteria may have on the growth, reproduction, and competitiveness of common and endangered legumes in their native habitats. Research proposed in this project is aimed at studying the metabolic potentials and host-specificity of rhizobia isolated from the nodules of common and endangered leguminous prairie plants in Illinois.

Astragalus crassicaarpus, an endangered plant species in Illinois, occurs in dry rocky prairies, glades, gravel prairies, open woods, and blufftops. This species was thought to be extirpated from Illinois until it was discovered in 1987 along a limestone bluff in Jersey County. This species was also rediscovered in Macoupin County. Neither population is protected (4). The reason why *Astragalus crassicaarpus* is endangered in Illinois is presently unknown. This study will focus on the symbiosis between *Astragalus crassicaarpus* and rhizobia, and how this relationship might impact the population size of *Astragalus crassicaarpus*. For example, the rhizobia that infect the roots of *A. crassicaarpus* may require special environmental conditions for growth and survival. Or, neighboring plants and their associated microbial populations that are needed to infect *Astragalus crassicaarpus* are rare. Also, the environment may prevent the *Astragalus crassicaarpus* - rhizobia symbiosis, and the plant only survives in areas where nutrients are available (e.g., when supplemented with fertilizer in runoff from agricultural fields). Hopefully this study will help shed some light on the nature of the interactions between rhizobia and *Astragalus crassicaarpus* as well as other common and endangered prairie legumes.

Objectives

The initial objectives of this study were: (i) to isolate and identify the rhizobia from the nodules of *Astragalus crassicaarpus* and from the nodules of common prairie legumes; (ii) to determine the metabolic diversity of rhizobia isolated from endangered and common legumes; and (iii) to assess the host specificity of rhizobia isolated from these endangered and common legumes. However, none of the prairie plants collected from the field site (Beaver Dam State Park in Macoupin County) possessed root nodules. Since the population of *Astragalus crassicaarpus* at this site is limited and since it is an endangered plant, extensive collecting of *Astragalus crassicaarpus* plants from this site was not performed. Thus, an alternative approach was developed and used.

The alternative approach involved the germination of seeds of endangered and common prairie legumes in sterile potting soil in the greenhouse and the application of soil (referred to as "bait soil") collected from prairie field sites across Illinois to young seedlings. These plants (referred to as "trap host species") were used to trap rhizobia present in prairie soils. With this alternative strategy, the objectives of the study were modified and included: (i) screening various bait soils collected from different various prairie field sites for rhizobia that can infect and nodulate the roots of endangered and common prairie legumes; (ii) determining the frequency of root nodulation of trap host species by microorganisms present in prairie bait soils; and (iii) isolating and identifying the rhizobia present in the nodules of *Astragalus crassicaarpus* and common prairie legumes used as trap host species.

Materials and Methods

Field Sites, Bait Soil Collection, and Sources of Leguminous Seeds. The main prairie field site for the collection of plants, soil, and seeds was located at Beaver Dam State Park near Carlinville, Illinois, in Macoupin County. This is one of only two dry gravel prairies in the state of Illinois known to contain *Astragalus crassicaarpus* (4). Soil was also collected from two other prairies sites within Illinois: Green River Conservation Area (a dry sand prairie located in Lee County) and Loxa Prairie (a rich loam, mesic prairie located in Coles County). From each site, soil samples (0-15 cm deep) were collected, transferred to a plastic container, sealed, and transported to the laboratory. All soils were stored at 4°C in the dark until subjected to analysis.

Seeds of *Astragalus crassicaarpus* were collected from Beaver Dam State Park. Seeds of *Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum* were purchased from Prairie Moon Nursery (Winona, MN).

Seed Preparation and Germination. Seed surfaces were sterilized by immersing seeds (10 at a time in strainer) in 200 ml of 10% bleach for two minutes. Bleached seeds were then rinsed three times in sterile water (200 ml per rinse). After sterilization, seeds were scarred by nicking them with a sterile scalpel. Before seeds per planted, pots (3-inch diameter) were sterilized in a 10% bleach solution as well as the eight trays containing the pots and allowed to dry. The pots were then filled 3/4 full with potting soil (a combination of vermiculite, fine sand, and gravel [1:1:1 ratio by weight] which had been autoclaved for one hour at 121°C). The soil was saturated with water and three seeds for a given plant species were added to a pot; 5 pots were used per plant species per bait soil type. The pots were watered with deionized water every other day, and N-containing 1/4 strength Hoagland's Nutrient Solution was added once a week. The N-containing 1/4 strength Hoagland's Nutrient Solution contained (milligrams per liter): K_2SO_4 , 109; $MgSO_4$, 60; $CaSO_4 \cdot 2H_2O$, 86; $CaHPO_4$, 71; KNO_3 , 1000; H_3BO_3 , 1.43; H_2MoO_4 , 0.009; $MnCl_2 \cdot 4H_2O$, 0.895; $CuSO_4 \cdot 5H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.11; and Fe-Chelate (Sprint 330), 25. Four weeks after germination, N-containing Hoagland's Nutrient Solution was switch to N-free Hoagland's Nutrient Solution (same as N-containing solution except the KNO_3 was omitted) and was used once per week for two weeks or until the bait soil was applied.

Bait Soil Studies. Soil collected from the three prairie sites (see above) was used as bait soil. A slurry of each prairie soil was made by mixing 10 g of soil in deionized water and applied to each pot at the rate of 5% of the volume of the pot. After adding bait soil, plants were watered daily, and, once per week for four weeks, received the N-free Hoagland's Nutrient Solution. Afer four weeks, plants were removed from potting soil. Root systems were examined for the presence of nodules as well as the extent of root nodulation.

Isolation and Identification of Rhizobia from Root Nodules. Nodules were removed from the plant roots, rinsed with tap water, surface sterilized in a 10% bleach solution for five minutes, and rinsed in sterile water. Surface-sterilized nodules were transferred to a sterile saline solution and crushed with a sterile glass rod. This material was then streaked onto yeast-extract mannitol agar (YEMA) plates and incubated at 25°C. YEMA contained (grams per liter): mannitol, 10; yeast extract, 0.2; NaCl, 0.1; $CaCO_3$, 0.01; congo red, 0.025; and agar, 15. With this medium, rhizobia appear as colorless or pinkish colonies while contaminants appear as bright red colonies. Colonies presumed to be rhizobia

(member of the genus *Rhizobium* or *Bradyrhizobium*) were restreaked on YEMA plates to check for purity. Presumptive rhizobial isolates were subsequently identified by standard microbiological and biochemical tests as outlined in the *Bergey's Manual of Determinative Bacteriology* [5].

Results

Field Studies. Root systems collected from endangered (*Astragalus crassicaarpus*) and common (*Lespedeza violaceae* and *Petalostemum candidum*) prairie legumes from several areas at the field site at Beaver Dam State Park did not possess nodules. This was unexpected and resulted in the development of an alternative approach (i.e., bait soil and trap host plants) to determine (i) if microbes present in prairie soils were able to nodulate endangered and common prairie legumes and (ii) what types or species of microbes were actually present in root nodules.

Preliminary Greenhouse Studies with Bait Soil from Beaver Dam State Park. When *Astragalus crassicaarpus* was inoculated with soil collected from Beaver Dam State Park, 14 of the 15 plants that were inoculated developed nodules on their root system (data not shown). These plants possessed between 1 to 3 nodules per root system. None of the control plants (those not inoculated with soil from Beaver Dam State Park) were nodulated.

Greenhouse Studies with Bait Soil from Different Prairies and Different Trap Host Plants. When *Astragalus crassicaarpus* was inoculated with soil collected from different prairie sites (Beaver Dam State Park, Loxa, and Green River Conservation Area), only microorganisms present in the Beaver Dam State Park were able to produce nodules on/in the roots of this endangered leguminous plant (Table 1). Interestingly, microbes present in Beaver Dam State Park were also able to nodulate *Petalostemum candidum* though nodulation was limited to one plant (Table 1). In contrast, microbial populations in Loxa and Green River Conservation Area soils generally failed to produce nodules on *Astragalus crassicaarpus* or on any of the more common prairie legumes (*Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum*) (Table 1).

Microbiological Studies. Nine isolates were obtained from the nodules of *Astragalus crassicaarpus* and one isolate from the nodules of *Petalostemum candidum*. Based on growth characteristics on YEMA plates, these isolates have been presumptively identified as rhizobia (either in the genus *Rhizobium* or *Bradyrhizobium*). Studies are presently underway to identify these isolates to the species level and to assess their host specificity with various prairie legumes.

Discussion

Why the endangered (*Astragalus crassicaarpus*) and common (*Lespedeza violaceae* and *Petalostemum candidum*) prairie legumes collected from the field site at Beaver Dam State Park were not nodulated is presently unclear. However, several possibilities exist which might explain the absence of nodules on these plants. First, it may be that not enough plants were collected or that not enough of the root system from each plant was collected; nodules are often at the tips (new growth) of roots and thus easily left behind when plants are extracted from soil. Another possibility could be that nodule development is

seasonal; in this study, plants were collected during the Fall and it may be that this was too late in the season. Evidence though to support this explanation is lacking in the literature. The age of the plant may have also been a factor; nodulation may only occur on young plants during the first few years of growth. What is certain is that the lack of nodulation was not due to the absence of nodule-producing microbes. Greenhouse studies demonstrated that microbial populations in prairie soil from Beaver Dam State Park were competent in the nodulation of the endangered legume *Astragalus crassicaarpus*.

The prairie at Beaver Dam State Park is unique in that it is one of only two prairies in the State of Illinois that harbor *Astragalus crassicaarpus* [4]. Of the three prairie soils tested, only microorganisms present in soil from Beaver Dam State Park nodulated greenhouse-grown plants of *Astragalus crassicaarpus*. It should be noted that seeds used in these greenhouse studies were collected from the prairie site at Beaver Dam State Park. In this regard, soils from prairies at Loxa and Green River Conservation Area failed to produce nodules on *Astragalus crassicaarpus*; both of these sites are not inhabited by *Astragalus crassicaarpus*. Collectively, these results indicate that the resident microbial flora in Beaver Dam State Park soil was specific for the host plant *Astragalus crassicaarpus*. Host plant specificity among strains and species of rhizobia is a trademark of this dynamic symbiotic relationship and has been well-documented over the years with wild and cultivated leguminous plants [6-8].

With the exception of one plant of *Petalostemum candidum* which developed nodules when exposed to Beaver Dam State Park soil, microbial symbionts in prairie soils from Beaver Dam State Park, Loxa, and Green River Conservation Area were generally deficient in their ability to nodulate common prairie legumes (*Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum*) in greenhouse studies. This was surprising for several reasons. First, Beaver Dam State Park soils contained active microbial symbionts for *Astragalus crassicaarpus* but yet apparently lacked symbionts for common legumes. Also, *Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum* are common to many prairies. Whether each resides in all three of the prairie sites examined in this study is being determined. However, *Petalostemum candidum* was found at the Beaver Dam State Park site. Interestingly, this was the only legume species other than *Astragalus crassicaarpus* that developed nodules when inoculated with prairie soil from Beaver Dam State Park. One explanation for the absence of root nodulation may be that the microbial symbionts for *Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum* were at low levels in the examined prairie soils (or not present at all) and thus were not in the bait soil that was added to the plants. Previous studies have shown that legumes may actually become symbiont limited due to a decrease in rhizobial populations during old field succession [9].

To date, numerous *Rhizobium* strains and a species of *Mesorhizobium* (*Mesorhizobium huakuii*, formerly *Rhizobium huakuii*) have been isolated from the plant genus *Astragalus*, and these organisms are quite diverse in their host and ecological ranges [10-12]. Whether the rhizobial microsymbionts isolated in the present study from *Astragalus crassicaarpus* are similar to other astragali rhizobia has yet to be determined. Studies to identify *Astragalus crassicaarpus* isolates are currently being conducted.

Little information is available relative to the diversity, physiology, ecology, and host ranges of native rhizobia in Illinois prairies. Information gained from this and future research will increase our understanding of native prairie legume-rhizobial symbioses and how this symbiosis impacts the distribution and densities of leguminous plant populations, especially endangered legumes. Hopefully, this will help us to define strategies in the future for the conservation and management of our native prairie plant populations.

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Table 1. Nodulation of Endangered (*Astragalus crassicaarpus*) and Common (*Amorpha canescens*, *Lespedeza virginica*, and *Petalostemum candidum*) Prairie Legumes with Soil from Different Prairies

Prairie Legume (Trap Host Species)	Prairie Soil (Bait Soil)	Number of Pots Containing Nodulated Plants (total number of pots examined)	Number of Nodulated Plants (total number of plants examined)	Average Number of Nodules Per Nodulated Plant
<i>Astragalus crassicaarpus</i>	Control	0 (4)	0 (6)	NA ^a
	Beaver Dam	4 (5)	8 (11)	5
	Loxa	0 (4)	0 (7)	NA
	Green River	0 (4)	0 (5)	NA
<i>Amorpha canescens</i>	Control	0 (4)	0 (4)	NA
	Beaver Dam	0 (4)	0 (5)	NA
	Loxa	0 (3)	0 (4)	NA
	Green River	- ^b	-	-
<i>Lespedeza virginica</i>	Control	-	-	-
	Beaver Dam	-	-	-
	Loxa	0 (1)	0 (1)	0 (1)
	Green River	-	-	-
<i>Petalostemum candidum</i>	Control	0 (4)	0 (7)	NA
	Beaver Dam	1 (3)	1 (3)	4
	Loxa	0 (4)	0 (6)	NA
	Green River	0 (5)	0 (6)	NA

^a NA, not applicable. None of the plants were nodulated.

^b No data was obtained because of high plant mortality. In these experiments, seeds germinated but none of these plants survived the duration of the experiment because of high temperatures which often occurred in the greenhouse during the Spring (2001) months.