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Competition between rhizobia under different environmental conditions affects the nodulation of a legume

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ABSTRACT

Mutualistic symbiosis and nitrogen fixation of legume rhizobia play a key role in ecological environments. Although many different rhizobial species can form nodules with a specific legume, there is often a dominant microsymbiont, which has the highest nodule occupancy rates, and they are often known as the "most favorable rhizobia". Shifts in the most favorable rhizobia for a legume in different geographical regions or soil types are not well understood. Therefore, in order to explore the shift model, an experiment was designed using successive inoculations of rhizobia on one legume. The plants were grown in either sterile vermiculite or a sandy soil. Results showed that, depending on the environment, a legume could select its preferential rhizobial partner in order to establish symbiosis. For perennial legumes, nodulation is a continuous and sequential process. In this study, when the most favorable rhizobial strain was available to infect the plant first, it was dominant in the nodules, regardless of the existence of other rhizobial strains in the rhizosphere. Other rhizobial strains had an opportunity to establish symbiosis with the plant when the most favorable rhizobial strain was not present in the rhizosphere. Nodule occupancy rates of the most favorable rhizobial strain depended on the competitiveness of other rhizobial strains in the rhizosphere and the environmental adaptability of the favorable rhizobial strain (in this case, to mild vermiculite or hostile sandy soil). To produce high nodulation and efficient nitrogen fixation, the most favorable rhizobial strain should be selected and inoculated into the rhizosphere of legume plants under optimum environmental conditions.

Introduction

Symbiotic nitrogen fixation with rhizobia is essential for the growth of legumes, especially in barren soils [4,33,39]. Some legumes, such as *Glycine max* [12,40,42], *Caragana* [9,41] and *Cicer arietinum* [14,26], can form nodules with different rhizobial species. However, most nodules are occupied by a few dominant microsymbionts, known as the favorable or highly competitive rhizobia [1,16,27,43]. In *G. max*, *Caragana*, *C. arietinum* and *Phaseolus vulgaris*, the species for favorable rhizobia shift depend on the geographical region and soil type [9,12,14,26,34,40–42]. For other legumes, such as *Medicago sativa* and *Trifolium* spp., the favorable rhizobia may even vary at the strain level [21].

Generally, during screening of the most effective nitrogen fixer for a legume, growth is assessed in a greenhouse using vermiculite followed by soil cultures, with final assessment in a field using local soils [10,11,37]. However, it is not clear how the competitiveness of favorable rhizobia shifts operates under different growth conditions, or what is the order of competitive nodulation for different rhizobial species. To understand the competitiveness of rhizobia and the competitive nodulation process, different rhizobial species that could nodulate a single legume were selected and the nodule occupancy rate of these species on a legume was assessed. The perennial legume *Caragana microphylla* can establish symbiosis with different rhizobia of the genera *Mesorhizobium* (the dominant microsymbionts) and *Rhizobium* [6,9,15,17–19,23,41], and it can grow in a broad range of soil environments, including

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Table 1
Growth substrate, inoculant and sampling strategy of *Caragana microphylla* for each treatment.

Code	Total plants	Treatments				Harvest time		
		Substrate[a]	1st inoculation[b]	Transplantation[c]	2nd inoculation[b]	45 d	90 d	135 d
I-A	20	Vermiculite	Control	No	Blank control	Yes	Yes	No
I-B	20	Vermiculite	CCBAU 01603	No	Mix (5)	Yes	Yes	No
I-C	20	Vermiculite	Mix (5)	No	CCBAU 01603	Yes	Yes	No
I-D	20	Vermiculite	Mix (6)	No	Mix (6)	Yes	Yes	No
II-A	20	Verm. + Soil	Control	No	Blank control	Yes	Yes	No
II-B	20	Verm. + Soil	CCBAU 01603	No	Mix (5)	Yes	Yes	No
II-C	20	Verm. + Soil	Mix (5)	No	CCBAU 01603	Yes	Yes	No
II-D	20	Verm. + Soil	Mix (6)	No	Mix (6)	Yes	Yes	No
III-A	10	Vermiculite	Control	Yes, in field	Blank control	No	No	Yes
III-B	10	Vermiculite	CCBAU 01603	Yes, in field	Mix (5)	No	No	Yes
III-C	10	Vermiculite	Mix (5)	Yes, in field	CCBAU 01603	No	No	Yes
III-D	10	Vermiculite	Mix (6)	Yes, in field	Mix (6)	No	No	Yes
CK_GH	10	Vermiculite	Control	No	No	Yes	No	No
MIX_GH	10	Vermiculite	Mix (6)	No	No	Yes	No	No
CK_Field	10	Soil in field	Control	No	No	No	Yes	No
MIX_Field	10	Soil in field	Mix (6)	No	No	No	Yes	No

[a] Vermiculite was sterilized; Verm. + Soil was a mixture of sterilized vermiculite supplied with 5 g natural soil in a water suspension (1 g mL^{-1}).
[b] For control, a sterilized 0.8% NaCl solution was used; Mix (5): mixture of strains *Mesorhizobium amorphae* CCBAU 01583, *M. temperatum* CCBAU 01582, *M. caraganae* CCBAU 01502, *M. silamurunense* CCBAU 01550^T and *M. septentrionale* CCBAU 01570 in the same volume. Mix (6): Mix (5) + *R. yanglingense* CCBAU 01603.
[c] Transplantation was or was not performed for the other 10 plants on the 45th day.

desert environments. In the present study, the competitive nodulation of *Mesorhizobium septentrionale*, *Mesorhizobium silamurunense*, *Mesorhizobium amorphae*, *Mesorhizobium temperatum*, *Mesorhizobium caraganae* and *Rhizobium yanglingense*, which can all nodulate *Caragana* [6,15,18,23,41], was assessed on *C. microphylla* under different growth conditions.

Materials and methods

Soil sampling and field trials

Soil samples were collected from the National Field Research Station for Ordos Grassland Ecosystems, Inner Mongolia, China, which is located in a semi-fixed desert (110°11′29″E, 39°29′37″N), where *Caragana* spp. are sporadically distributed. The soils were characterized as sandy soils with pH 8.62.

Field trials were carried out at the research station. The field trial area (10 × 12 m) was covered with wild vegetation, such as *C. microphylla*, *Caragana intermedia*, *Hedysarum mongolicum*, *Artemisia songarica*, *Psammochloa villosa*, and *Astragalus melilotoides*, as described previously [15]. The field trial area was divided into six plots, according to the treatment design (Table 1). No fertilizer was used before or during the experimental period and the soil was irrigated every fifteen days, to ensure there was adequate moisture for plant growth.

Representative strains and preparation of inoculants

Strains known to nodulate *C. microphylla* well were used (*R. yanglingense*, CCBAU 01603; *M. amorphae*, CCBAU 01583; *M. temperatum*, CCBAU 01582; *M. caraganae*, CCBAU 01502; and *M. septentrionale*, CCBAU 01570). Each of these strains represented a different major group (they clustered separately based on their rDNA type [15,18]) and fixed nitrogen efficiently on the roots of *C. microphylla*. *M. silamurunense* (CCBAU 01550^T), isolated from the roots of *Astragalus membranaceus* [44], was also used as an example of a relatively inefficient rhizobial nitrogen fixer on the roots of *C. microphylla*. Each strain was isolated from the National Field Research Station for Ordos Grassland Ecosystems and was stored in the Culture Collection of Beijing Agricultural University (CCBAU).

To prepare the inoculants, each strain was cultured in 4 mL of tryptone yeast (TY) broth [29] at 28 °C with shaking (180 rpm) until the logarithmic phase was reached [43]. Then, each culture was transferred into 50 mL fresh TY broth and incubated under the same conditions to an OD_{600} of 1.5. Pellets were obtained by centrifugation of the cultures and they were then re-suspended to an OD_{600} of 1.0 in a sterilized 0.8% NaCl solution that represented 10^8 CFU/mL. The resuspension of each culture was used as a single inoculation for the symbiotic efficiency tests. For the competitive nodulation assays, three treatments were used: (1) strain CCBAU 01603 alone; (2) a mixture of the five mesorhizobial strains, Mix (5); and (3) a mixture of all six strains, Mix (6). The inoculants for the competitive nodulation assays were prepared in equal volumes of cell suspension. For the control experiment, 0.8% NaCl solution was used.

Seed germination and greenhouse conditions

Seeds of *C. microphylla* were treated with 95% alcohol for 40 s, surface sterilized with 2.4% NaClO for 8 min, and then rinsed seven times with sterilized water [35]. The sterilized seeds were pre-germinated on a 0.7% water-agar medium at 28 °C for 48 h in darkness [8].

To grow the *C. microphylla* seedlings, double-layer Leonard jars filled with vermiculite, moistened with a low-nitrogen nutrient solution [35], were sterilized at 121 °C for 90 min. After sterilization, 5 g soil samples suspended in sterile water (1 g mL^{-1}) were added and mixed with the sterilized vermiculite in each jar in order to produce the "Verm. + Soil" treatments (Table 1). One pre-germinated seed was sown in each jar (Table 1 and Fig. S1). The seedlings were inoculated, according to the treatment design, with an inoculant or a NaCl solution (0.8%) (1 mL per seedling) (Table 1 and Fig. S1). The plants were cultured in a greenhouse, with a day/night cycle of 3000 Lx illumination for 16 h at 25 °C and darkness at 23 °C for 8 h.

Analysis of symbiotic effectiveness

To assess the symbiotic effectiveness of the rhizobia, inoculation tests with each individual inoculant were carried out in sterilized vermiculite in the greenhouse. The plants were harvested 45 days later, and then nodule numbers, fresh nodule weights, shoot heights and shoot dry weights were recorded. All results are reported as the mean ± standard error (SE) of three replicate groups, and data were subjected to one-way analysis of variance (ANOVA). When there were significant differences ($P < 0.05$), the