Studies on the Characteristics Related to Symbiotic Nitrogen Fixation of Legumes in Xinjiang Region

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Abstract: 1208 samples of root nodules of legumes belong to 31 genera, 109 species in Xinjiang arid area were collected. The nodulation of 39 species has not been reported. 95% of the nodules formed by rhizobia were efficient in nitrogen fixation. The differences of the nitrogenase activity existed in all kinds of nodules obviously. The nodules from *Astragalus* possessed high activity of nitrogen fixation and the maximum of which was 42 times more than that of soybean nodules in same locality. Uptake hydrogenase activity in nodules was detected in 97% of 37 species from 20 genera. Both nitrogenase and hydrogenase activities were closely related to the growth of plants. The characteristics related to symbiotic nitrogen fixation and action of soil and water conservation in environment were discussed.
Keywords: root nodules bacteria, symbiotic nitrogen fixation, soil and water conservation

1 Introduction

The Xinjiang autonomous region is situated at 36° — 48° N and 71° — 96° E in the heart of Eurasia. Its landform is complicated because surrounded by high mountains. Rainfall of the region is rare, but evaporation is high, so the climate is dry. The temperature variations are great between day and month. It is a typical inland arid region. Since affected by topographic features, many different ecological conditions are formed. Desertification and salinization of the soil is widespread because of dry climate. However, in this great natural arid ecological system there are various kinds of N_2-fixing microorganisms which have adapted to the conditions and possess distinctive genetic characteristics of dry and salt resistance. Xinjiang has a vast territory of about 1.65 million square kilometers, but the cultivated land only accounts for about 2% of the whole area[1]. The proportion of available nitrogen applied to the environment through fertilization is obviously low. Through biological nitrogen fixation, atmospheric nitrogen is transformed into available nitride and becomes the main sources of available nitrogen, so nitrogen fixation by microorganisms including the symbiosis of legume-rhizobium plays a very important role in nitrogen cycle of the ecosystem. The research and application on rhizobia in Xinjiang not only can improve the soil fertility and increase crop productivity but also is significant to the consumption of energy and environmental protection. According to the statistics by Allen and others, the species of legume in the world, which have been studied and have symbiosis with rhizobia, are less than 0.5%. Most of the rhizobia are still unknown [2,3]. The study of rhizobium sources in arid areas and the isolation of new species or strains will certainly provide scientific information for the classification of rhizobia. In this work, we have tested nitrogen fixation ability, resistance to unfavorable conditions and biochemical characteristics of legume-rhizobium symbioses.

2 Materials and methods

2.1 Collection of root nodules

Root nodules were collected from the natural plains in Zhaosu, Tekesi, South Lake in Tacheng and Hanasi Lake in Aertai, etc, which are scattered over the area of Xinjiang Yili. The nodules of arenaceous legume were collected from the dunes in Huocheng, Mulei in the east of Xinjiang, Qitai, Mosuowan and

Turpan in the brink of Zhunger Pan. The nodules of fruticose legume were sampled from Baiyanggou in Manasi,Nansan in Ürümqi and Tianchi, which are located in the vicinity of Tianshan mountain forest area. The nodules of ephemeral legumes were gotten from Miquan, Chabuchaer and Cangfanggou. In addition, the nodules of legume crop were collected from Shihezi and Xiayedi in the north of Xinjiang, Kashi, Akesu and Kuche in the south of Xinjiang. Root nodules were obtained from budding or flowering legume. The samples were kept intact, and the quantities of the samples were as large as possible. After scouring, the samples were kept in 8ml bottles.

2.2 Determination of the acetylene reduction activities of the nodules

This test was finished in accordance with the advanced method of the ratio of gas chromatograph peak height between ethylene and acetylene designed by The Laboratory of Nitrogen fixation of The Shanghai Institute of Plant Physiology[4] .

2.3 Determination of hydrogen-uptake activities and hydrogen-evolution of nodules

The nodules were cleaned and put into 8ml bottles closed with rubber stoppers. The hydrogen-evolution of samples which had grown for 48 hours at 28℃ were determined. The sampled nodules after 48 hour starvation were closed in 8ml bottles in which pure hydrogen was injected. After placing at 28℃ for 48 hours, the hydrogen-uptake of the samples were determined by domestic 103 gas chromatograph analyzer, with a molecular sieve 5A column and nitrogen as carried gas. The Column pressure was 0.4 kg/cm^2—$0.6 kg/cm^2$ and the electric current detector was set to 70mA [5] .

3 Results and discussions

3.1 The nitrogen fixation derived from the legume-rhizobium symbiosis

The nodule is formed via symbiosis between the rhizobium and its host plant. Under suitable circumstances, soil rhizobia and plants can recognize each other. Because of the stimulus of the exudates from the leguminous root, rhizobia propagate rapidly and pass through the epidermis tissue to form infectious threads. In the meantime they promote cell division of the root tissue to form nodules gradually. The shapes of leguminous nodule are varied in the samples collected in the Xinjiang arid area. After entering the host cells, the rhizobia change into bacteria's and fix N_2. Infecting legumes and forming nodules for nitrogen fixation is the remarkable characteristic of rhizobia which is different from the other bacteria in the soil. In order to prove the symbiotic N_2 fixation of the legume-rhizobium in Xinjiang, more than 1,000 samples were collected from over 100 species of budding/ flowering legume and the nitrogen fixing activities of these nodule samples were detected with the acetylene reduction method[6]. The results showed that 95% of the nodules are effective for nitrogen fixation but the activities in different species varied greatly (Table 1). *Astragalus* generally possessed high activities, the maximum was up to 365.00 μ mol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$,42 times more than that of *Glycine max* nodules under the same condition. The nodules of some other legumes especially grew in the arid area, such as *S.alopecuroides*, *H.mongolicum*, *C.intemedia* and a short-lived plant *T.cancellata*, also possessed higher fixation activities (more than 20 μ mol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$). *Trifolium* genus plants, which was widely distributed in the world, were also found in Xinjiang. The root nodule of *T.repens* growing in the natural South Lake grassland was pink and showed high fixation activities, up to 523.90 μ mol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$. 60 times more than that of *Glycine max*.

The factors affecting the nitrogen fixation activity of nodules are very complex. They are mainly due to genetic properties and environmental conditions. Energy, which is necessary for the bacteroids in nodules for survival and for N_2-fixation, is supplied by the plants photosynthesis. Therefore, the nitrogen fixation of the nodules is certainly regulated by the host plant.

Table 1 The Acetylene Reduction Activity in Some Symbiosis Nodules of Leguminous Plant-Rhizobium in The Xinjiang Arid Area

Host plant	Sampling place and conditions	C_2H_2 reduction activity (μ molC_2H_4 • g^{-1} • h^{-1})
Astragalus adsurgens	Mosuowan sands	63.23
Astragalus grandiflorus	Huocheng sand dune	19.12
Astragalus hypogaeus	Yili sand dune	16.78
Astragalus karkarensis	Tacheng grassland	365.53
Astragalus lasiophyllus	Urumqi desert hill	121.70
Astragalus lehmannianus	Urumqi desert hill	26.88
Astragalus oxyglottis	Fukang sand dune	29.98
Astragalus steinbergianus	Qitai sand dune	17.56
Astragalus stenoceras	Chabuchaer desert	119.81
Astragalus suiduensis	Huocheng sand dune	16.78
Alhagi sparsifolia	Turpan sands	2.48
Ammopiptanthus mongolicus	Turpan sands	12.63
Caragana intermedia	Turpan sands	113.40
Cicer arietinum	Urumqi farmland	1.17
Caragana jubate	Turpan sands	10.64
Eremosparton songoricum	Qitai sand dune	10.31
Halimodendron halodendron	Mosuowan sands	10.25
Halimodendron mongolicus	Turpan sands	23.99
Hedysarum alpinum	Manasi forest	3.33
Glycine max	Shihezi farmland	8.75
Lathyrus humilis	Mosuowan sands	15.66
Onobrychis sp.	Qitai grassland	5.58
Pisum sativum	Mosuowan farmland	0.10
Sophora alopecuroides	Shihezi farmland	0.06
Sphaerophysa salsula	Yili desert	28.27
Trifolium pratense	Tacheng grassland	65.91
Trifolium repens	Tacheng grassland	523.90
Trigonella cancellata	Miquan desert hill	29.98
Trigonella foenum-graecum	Turpan farmland	85.98
Vicia sepium	Tacheng grassland	46.64
Vicia villosa	Urumqi farmland	0.90
Vigna sinensis	Turpan farmland	0.24
Medicago falcata	Manasi forest	83.64

According to Bergenson, 30%—40% of the whole nitrogen fixation was close to the flowering period in *Glycine max*[7]. The observation of nitrogen fixation abilities during the different stages of plant growth were carried out on one kind of short-lived plants, *T. fenuis*, growing in natural condition and three artificially cultivated plants, *V. villosa*, *C. arietinum* and *M. sativa*. The nodule samples were collected in every growth period and their nitrogen fixation ability was determined. The results were summarized in Table 2. It appeared that the activity of the nodules were not only closely related to growth periods, but also to plant habitats. The maximal activity appeared in bud. The nitrogen fixation activities of *T.fenuis* nodules decreased along with the plant growth. The activity during flowering was only 30% of that during budding. Perennial *M.sativa* nodules maintained activity in every growth period.

The expression of nitrogenase in bacteroids was regulated by many factors such as microenvironment and substance transportation in the nodules [8]. There are changes of metabolic processes and regulation of oxygen concentrations in the nodules excised from the host plants. It means that a notable impact on the expression of the nitrogenase activity exists in the bacteroids. Nodule samples were selected during the flowering of *T. foenum-graececum*, *Vicia sp*. and *C.arietium* and acetylene reduction activities were tested for every hour. The results showed that the nitrogenase activities of the excised nodules from different kinds of plants had their own peak time . The maximum of nitrogenase activity in *T.foenum-graeceum* nodules appeared in the third hour, by *C.arietinum* in the sixth, by *Vicia sp*. in the fifth.

The nitrogen fixation in the legume-rhizobium symbiosis nodules is the key view of symbiotic nitrogen fixation research. Moreover, it is the phenomenon much noticed by people. Various factors are involved in the nitrogen fixation activity of rhizobium. In addition to the hereditary features of rhizobia themselves, the activities are also regulated and controlled by the physiological metabolism of the host plants. In terms of ecology, the soil water content of arid areas is an important factor that affects the nitrogen fixation activity of rhizobium. *Trigonella arcuata* nodules were sampled in the same growth sites and having the same growth periods and the acetylene reduction activities were determined. The soil humidity was 15.0% (0 cm—20 cm) and the nitrogen fixation activities of the nodules were 16.62μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$. Another year, the soil humidity was 11.7% (0 cm—20 cm) and the nitrogen fixation activity of the nodules was reduced to 3.34 μ mol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$. After the determination of the nitrogen fixation activities of the nodules, accumulations of acetylene reduction was generally determined 1 hours-2 hours after nodule isolation. The results showed that nitrogen fixation activities of different nodules varied after 1 hours-2 hours. And the activity had a peak within a certain time. Therefore, it is noticeable that the time of acetylene reduction activity was determined in the research of the nitrogen fixation in legume-rhizobium symbiosis. It will be further quested that a peak of acetylene reduction activity appeared after the nodule isolation.

Table 2 The relationship between C_2H_2 reduction activity of nodules and growth stages of plants

Host plant	Living habits	C_2H_2 reduction activity (μ mol$C_2H_4 \cdot g^{-1} \cdot h^{-1}$)			
		In budding	In early flowering	In flowering	In podding
Trigonella fenuis	short-lived plant	25.62	19.84	7.80	0.14
Vicia villosa	annual plant	19.14	14.34	8.10	0.27
Cicer arietinum	annual plant	3.84	3.00	10.98	0.08
Medicago sativa	perennial plant	16.62	16.32	14.46	13.32

*The living period of these plants is on March to May only.

3.2 Hydrogen-evolution and uptake in the legume-rhizobium symbiosis nodules

It was testified that the nitrogenase *in vitro* from various species of nitrogen fixing organisms catalyzes ATP-dependent hydrogen evolution in the process of nitrogen fixation, 25%—30% of the electrons flowing to the nitrogenase was wasted in H^+ reduction. Hydrogen evolution via nitrogenase may consume up to 40%—60% of the energy supplied to the nitrogen fixing process in legume nodules. However, uptake of hydrogenase activity was found in legume nodules formed by some rhizobia. The hydrogenase can recycle the hydrogen produced by nitrogenase. The oxygenation of hydrogen can reduce partial oxygen pressure to protect nitrogenase[6,,7]. According to Evans[8], most strains of *R.japonicum* did not carry uptake hydrogenase genes in their cells, a few strains of *R.meliloti*, but most strains of *R.vicia* possessed the genes. In our lab the hydrogenase activities of nodules from 37 species belong to 20 genera of legumes were detected as described by Evans[12]. Only the nodules of *A.ammodytes* were unable to uptake hydrogen, and the others had the ability of hydrogen uptake activity, H_2 uptake of the nodules species were below 1 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$ in once experiment, by 14 species 1 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$—5 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$,by 10 species 5 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$—10 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$, by 9 species more than 10 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$.The highest activity was up to 68.65 μ mol $H_2 \cdot g^{-1} \cdot h^{-1}$. This showed that most rhizobia in Xinjiang including *R.meliloti* and *R.trifolii* possess uptake hydrogenase. The nodules formed by these rhizobia release little or no hydrogen into the air, supplying a useful additional energy source for symbiosis nitrogen fixation. So they are more efficient in utilization of photosynthesis products.16 species of nodules tested here could evolve hydrogen, one of them *A.ammodytes*, lacked the hydrogen uptake activity, the amount of hydrogen evolution was up to 7.26 μ mol H_2 • g^{-1} • h^{-1}. The efficiencies of nitrogen fixation in nodules from 20 species of plants were calculated with Evans' formula (Table 3)[8]. It showed that the values of the most relative efficiencies were equal.

The uptake hydrogenase genes in rhizobia may be expressed in free-living conditions, but the medium and incubation conditions should be selected exactly[7]. The uptake hydrogenase expression of bacteriods in nodules was affected by nodule microenvironment and regulation of plant growth. The uptake hydrogenase activities of the nodules from 6 species were measured at every stages, the maximum activity existed during budding and the minimum during potting (Table 4). However, the positive relativity between hydrogen uptake activity and nitrogen fixation activity could be found during different growth periods in nodules from T.arcuata which only grew in arid areas (Table 5).

Table 3 Hydrogen-uptake activities and relative efficiency of nitrogen fixation of legumious nodules

Host plant	H_2-uptake activity (μ mol H_2 • g^{-1} • h^{-1})	H_2-evolution activity (μ mol H_2 • g^{-1} • h^{-1})	C_2H_2 reduction (μ mol C_2H_4 • g^{-1} • h^{-1})	Relative efficiency
Orobus luteus	7.19	0	1.61	1
Lathyrus hmilis	6.28	0	29.28	1
Vicia cracca	15.65	0	3.24	1
Hedysarum austrosibiricum	6.35	0	1.11	1
Trifolium lupinaster	7.09	0	7.14	1
Thermopsis kuelunice	3.76	0	6.42	1
Halimodendron halodendron	3.89	0.04	2.82	0.98
Medicago falcata	3.12	0	3.18	1
Cicer arietinum	0	3.84	5.44	0.29
Melilotus officinalis	14.22	0	4.72	1
Glycine max	0.47	0.35	0.47	0.25
Crotalaria juncea	3.93	0	ND	ND
Trigonella arcuata	68.65	0	52.64	1
Alhagi pseudalhagi	4.56	0	3.12	1
Oxytropis sp.	4.59	0	5.40	1
Glycyrrhiza glabra	0.70	0.07	ND	ND
Sphaerophysa salsula	0.78	0	1.32	1
Caragana intermedia	28.54	0	48.18	1
Onobrychis tanpitica	12.27	0	1.16	1
Astragalus stenoceras	7.48	0	10.98	1

ND: Not determined.

Table 4 The relationship between hydrogen-uptake activities of leguminous nodules and growth periods of plants

Host plant	H_2-uptake activity(μ mol H_2 • g^{-1} • h^{-1})		
	In budding	In flowering	In podding
Trigonella arcuata	68.65	21.26	1.88
Lathyrus quinguenervius	9.33	0.18	0.04
Onobrychis sp.	1.33	0.54	0.09
Cicer arietinum	2.16	0.76	0.09
Vicia sativa	4.31	1.61	0.22
Glycine max	4.73	0.23	0.03

Table 5 Hydrogen-uptake activities and acetylene reduction activities of *trigonella arcuata* nodules in different growth periods

Growth periods	H_2-uptake activity (μ mol H_2 • g^{-1} • h^{-1})	H_2-evolutionactivity	C_2H_2 reduction activity (μ mol C_2H_4 • g^{-1} • h^{-1})	Relative efficiency
In budding	24.55	0	52.64	1
In flowering	21.26	0	30.28	1
In podding	1.88	0	7.38	1

Some rhizobia with uptake hydrogenase genes can grow in autotrophic conditions, but are restricted in the choice of condition environment and medium components.[9] Our results showed that over 98% rhizobia in Xinjiang arid area possessed uptake hydrogenase using media and hydrogen induction protocol according to Maier. Only *Rhizobia vicia sp.* from more than 100 strains of rhizobia possessed the uptake hydrogenase expression activity in the autotrophic conditions. Furthermore, its sensitivity to carbohydrates and all kinds of ions are all lower than those of *Rhizobia astragalus sp*.[9]. One characteristic of the arid area is evident variation of temperature, which is beneficial to the accumulation of carbohydrates. The carbohydrate accumulation of the legume nodules in this area is probably larger than that of other areas. It may be not fit for the expression of rhizobium uptake hydrogenase activities when rhizobia were cultivated in low carbon level.

3.3 The application prospect of rhizobium bacteria in xinjiang

It was clear that the rhizobia in Xinjiang have highly resistance to unfavorable conditions and can grow well in this soil. We naturally selected the strains with higher activities of nitrogen fixation, stronger infectious activities and resistant to disadvantages by means of tube and basin plant cultures. These strains were inoculated into leguminous plants subsequently in the field. The results showed that the grass production of *Medicago*, *Glycyrrhiza glabra* and *Glycine max* increased 30%, 40% and 10% respectively. Therefore the rhizobia resource in the Xinjiang arid region will play an important role in agriculture. In addition, we noticed some of the rhizobia in Xinjiang could produce pink and golden yellow pigments, especially a large amount of mucopolysaccharides and low temperature proteinases. These products may be useful in the fermentation industry during food and enzyme productions. 373 strains isolated from 109 species of legumes distributed in Xinjiang were the representatives of the whole rhizobia populations in this region. Many strains among them were recorded for the first time, which greatly enriched the rhizobia resources in the world. The discovery of the new strains of rhizobia must have important value in the taxonomy of rhizobia. Our experiments proved that all the rhizobia source in Xinjiang possessed distinct physiological, biochemical and ecological characteristics. These characteristics reflected that they possessed distinctive genetic properties, so they were valuable materials in the research of molecular biology and the genetics of nitrogen fixation.

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