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Screening of high effective alfalfa rhizobial strains with a comprehensive protocol

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Abstract - Ninety-six alfalfa rhizobial strains were screened with a two-step strategy to select high effective and competitive microsymbionts for this plant grown in Inner Mongolia. Firstly, all the strains were inoculated to surface-sterilised seeds sown in vermiculite and grown in greenhouse. After grown 45 days, the shoot dry weight, number and nitrogenase activity of nodules were recorded and statistically analysed to select the more effective strains. Secondly, the competitiveness of 4 more effective strains designated in first step were evaluated by inoculation of each strain to alfalfa seeds grown in natural soil collected from Inner Mongolia. The nodule occupancy of each inoculated strain was assayed with BOX-PCR fingerprinting. With this strategy, 4 strains, all isolated from the same eco-region, were claimed as the best symbionts with alfalfa in that soil. The statistically designated best strains were exactly the most competitive and most effective ones in the second step, and the results were further confirmed by a field experiment. This study offered an efficient strategy to select rhizobial inoculants and demonstrated the possibility that the local rhizobial population might be highly effective and competitive for the epidemic legumes there.

Key words: screening, BOX-PCR fingerprinting, effective symbiont, alfalfa, rhizobia.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is believed to be the only forage crop that was cultivated before the recorded history (Allen and Allen, 1981). This plant was induced from ancient Kashmir into China around 126 AD by Mr. Qian Zhang (about 164 to 114 AD), an ancient Chinese diplomat who explored the Silk Road. Up to date, alfalfa has become one of the most important forage plants in the northern parts and many cultivars adapted to the local conditions have been obtained in China. Although same as in other region (Kuykendall *et al*., 2005; Silva *et al*., 2007), alfalfa nodulates with *Sinorhizobium meliloti* in China, the alfalfa rhizobial isolated from Chinese soils had phenotypic or genetic characteristics different from those originated from other countries (Yan *et al.*, 2000; Kan *et al*., 2007), demonstrating that the compatible rhizobial have evolved in association with the alfalfa cultivars grown in different eco-regions in China, or they were indigenous to the Chinese soils.

Different from the cultivation in New World countries, such as the USA, Canada, and Australia, the alfalfa seed inoculation with rhizobial is rare in China, a part of the Old World. In relation to the enlarged cultivation of alfalfa and the emphasis of sustainable cultivation in China recently, inoculation of rhizobia has been accepted by more and more Chinese farmers. Considering the increased demand of inoculants and the estimation that nodule symbiosis is a result of interaction among the host legume, the rhizobia and the environmental factors (Han *et al.*, 2005), some studies about the high efficient strains have been performed in China (Zeng *et al*., 2004; Zhao *et al*., 2005; Yang *et al*., 2006). A separate inoculation of 10 *S. meliloti* strains could increase 80-250% (with average of 216%), 16.30-51.0 (2.1%) and 20.41-66.70% (with average of 29.9%) of alfalfa dry weight in three different cultivars (Zhao *et al*., 2005), and 10-13% or lower in another experiment with 18 strains against the cultivar Vector (Canada) (Zeng *et al*., 2004). Therefore, it was clear that each alfalfa cultivar could select a subpopulation of *S. meliloti* as its suitable symbiotic partner. The specificity among legume cultivars-rhizobial subpopulations might be determined by the legume genotype and rhizobial host specific genes as revealed in *Pisum sativum* cv. Afghanistan (Firmin *et al*., 1993).

In China, the alfalfa cultivated areas are divided into three ecological regions according to the winter hardiness of alfalfa cultivars grown there (Lu, 1998). The first eco-region included the northern Xinjiang and the central and western zones of Inner Mongolia where the altitude is 1000-2000 m; annual average temperature is 5-9 °C; annual precipitation is 50-250 mm; the winter is very cold and the summer is very hot. The second eco-region refers to the southern Xinjiang, where is very dry and hot in the growing season, with dramatic change of temperature in day/night time, and strong solar radiation. The other eco-region is intermediate type including the other Chinese provinces in

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temperate region, where has warmer and semiarid climate. Alfalfa cultivars with different morphologies and adaptation features have been found in the three eco-regions.

Inner Mongolia has the second biggest meadow in China and alfalfa is a popular cultivated forage plant there. Except the native cultivars, some cultivars originated from other eco-regions or countries have been introduced into that region. In addition, the evaluation of effectiveness and competition of the rhizobial inoculants associated with the cultivars grown there is rather insufficient. The object of this study was screening the high effective and competitive rhizobial adapted to soils of Inner Mongolia with a rapid and effective protocol.

MATERIALS AND METHODS

Strains and their geographic origins. Ninety-six test rhizobial strains were obtained from the Culture Collection, Beijing Agricultural University, China (CCBAU) and they were stored in 40% (w/v) glycerol at -80 °C. These strains were isolated from nodules of *Medicago sativa* L.*, Medicago falcata* L.*, Medicago lupulina* L.*, Medicago polymorpha* L. and *Medicago archiducis-nicolai* Sirj grown in the northern regions of China, including provinces of Inner Mongolia, Shaanxi, Shanxi, Qinghai, Jilin, Gansu, Heibei, and Xinjiang (Table 1). The strains were firstly activated by striking in YMA medium (pH 7.0-7.2) supplied with 0.02% BTB (Bromothymol blue) as pH indicator (Vincent, 1970) and incubated at 28 °C. The purity of each strain was checked by observation of colonial morphology and cellular morphology with Gram-staining. Production of acid or alkali was recorded from the colour change of BTB.

Screening step I (effectiveness assay in vermiculite).
Inoculation. In this analysis, seeds of *M. sativa* cv. Aohan were used because this cultivar is native to Inner Mongolia and is one of the main alfalfa cultivars grown in the temperate region of China. The seeds were surface sterilised following the method of Vincent (1970) and were sown in vermiculite moisturized with low-N nutrient solution (Vincent, 1970) in Leonard jars (Vincent, 1970; Bromfield, 1984). In each jar, 2-3 seeds were put and germinated in dark at room temperature for 48 h. Then the germinated seeds were inoculated separately with the 96 test strains (10^8 CFU/jar), and the blank controls were inoculated with sterilised water. The test strains were incubated in TY broth (tryptone 5 g, yeast extracted 3 g, $CaCl_2$ 0.7 g in 1000 ml deionised water, pH 6.8-7.2) with shaking at 28 °C for 48-72 h and the concentration was controlled at OD_{500nm} = 0.5 (equal to 1 x 10^8 CFU/ml). The experiments were performed in triplicate under natural sunlight and temperature in greenhouse, 14-16 h in daytime at 24-28 °C and 8-10 h in night time at 18-22 °C. All the bottles were arranged in randomized blocks, disarranged in every two days to avoid the variance caused by illumination difference, and watered with sterilising deionised water when it is necessary.

Biomass production and acetylene reduction assay. After 45 days of inoculation, the dry weight of shoots (DWO), total number of nodules (TNN), and activity of nitrogenase (acetylene reducing activity, ARA) were assayed. The shoots were cut and dried at 65 °C to constant weight and weighted. The roots were washed to eliminate attached vermiculite, soaked up on paper towel and then shifted into 100 ml serum bottle sealed with silica gel stopper for acetylene reduction assay immediately as described by Staal *et al*. (2001).

The nitrogenase activity was measured as following: drawn out 10 ml air from the serum bottle with roots, injected into 10 ml pure acetylene, incubated at 28 °C for 1 h. Then 100 µl gas sample was taken from the serum bottle and was injected into Hewlett Packard GC system (HP 6890 series, Hewlett Packard, INC, USA) for gas chromatograph analysis. The conditions of GS configured as follow: injector temperature: 120 °C, with capillary column of HP plot / Al_2O_3 15 m x 0.53 mm x 15 µm film thickness (USA), ultra high purity N_2 as carrier, with column flow 1.5 ml/min, oven temperature 70 °C, FID detector at temperature 250 °C, H_2 flow 40 ml/min, air flow 300 ml/min, makeup (N_2) flow 28.5 ml/min. The amount of ethylene calculated with the formulas F1 and F2:

F1 $V = K \times C \times$ peak height ratio (C_2H_4/C_2H_2)

where V is the volume of ethylene, K is the reciprocal related coefficient of peak height ratio (C_2H_4/C_2H_2), C is the volume of acetylene injected;

F2 $N = V \times 1/22.4 \times 273/(273 + t\ °C) \times P/760 \times 10^6$

where N presents nanogram molecule C_2H_4 at the standard atmospheric pressure (760 mmHg); t is 25 °C. The unit of nitrogenase (U) was defined as nanogram molecule C_2H_4 per hour per plant (root).

Screening step II (competition assay in soil).
Inoculation. The soil used in this study was sampled from Institute of Horticulture of Inner Mongolia (Hohhot, capital of Inner Mongolia, 669 km north of Beijing). This soil was loam with 2.3% of organic materials, 0.123% of total nitrogen, 1.4 mg/kg alkali-hydrolysable N, 41 mg/kg available phosphorus (P), 144 mg/kg available potassium (K), pH 7.6. The mostly probability number of indigenous alfalfa rhizobial in this soil was about 100 CFU/g soil estimated by the plant trapping method (Vincent, 1970) (data not shown).

In this assay, alfalfa seeds without surface sterilisation were sown in Leonard jars (2-3 seeds each bottle) half filled with natural soil. Five duplicates were included. After two days for germination, 1 x 10^8 CFU of rhizobial culture was inoculated to each jar. The germination, inoculation and naturalisation of green house were the same as mentioned in screening step I, except that tap water was used instead of low-N nutrition and sterilising deionised water.

Estimation of nodule occupation by inoculated strain with BOX-PCR. Nodules of each plant grown in the soil were counted and collected in 1.5 ml microtubes on the 50^{th} day after inoculation. Twenty randomly selected nodules from one plant in each duplicate were surface sterilised and were used in bacteria isolation using YMA medium and protocols described by Vincent (1970). The isolates obtained from nodules were then incubated in TY to logarithmic phase for total DNA extraction following the method of Chun *et al*. (1999). DNA samples were stored at -20 °C until use.

BOX PCR was performed in 25 µl reaction mixture with each DNA sample obtained from the nodule isolates by using BOX-A1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) and corresponding protocol (Laguerre *et al*., 2003). Electrophoresis was carried in 2.5% agarose supplied with 0.5 µg/ml ethidium bromide. The PCR fingerprinting was photographed and saved as TIFF format with Alpha Innotech multimage (Alpha Ease FC v4.1.0, Alpha Innotech Corporation) after the electrophoresis. The Gelcomp II software (v4.50, Applied Maths) was used to define the BOX PCR fingerprinting type and to cluster the isolates according to the BOX PCR patterns with Dice coefficient and UPGMA (unweighted pair group method with arithmetic mean)

TABLE 1 - Rhizobial strains used in this study

Strain (CCBAU no.)*	Eco-region	Host species	Reference or geographical origin
Sinorhizobium meliloti			
03041	III	*Medicago sativa* L.	XM Cai (Thesis)
83065, **83066**, 83068, **83085**	II	*Medicago sativa* L.	Yan *et al*. (2000)
83164, **83168**, **83178**	I	*Medicago sativa* L.	Yan *et al*. (2000)
Unclassified strains			
01001, 01135, 01187, 01262	I	*Medicago falcata* L.	Inner Mongolia
01006, 01034, 01132, 01192, 01264, **01290**, 01291, 01298, 01300, 01301, 01302, 01306, 01307, 01308, 01309, 01310, 01311, **01314**, 01315, 01316, 01317, 01325, 01326, 01327, 01349, 01352, 01353, 01354, 01355, 01356, 01360, 01415, 01416, 01417	I	*Medicago sativa* L.	Inner Mongolia
01013	I	*Melilotus alba* Medic ex Desr.	Inner Mongolia
01019, 01028, 01059, **01199**, 01236	I	*Medicago lupulina* L.	Inner Mongolia
01280	I	*Melilotus officinalis* (L.) Desr.	Inner Mongolia
03009	III	*Medicago lupulina* L.	Shanxi
03035	III	*Medicago falcata* L.	Shanxi
05015, **05188**	III	*Medicago sativa* L.	Hebei
05057, **05180**, **05210**	III	*Medicago lupulina* L.	Hebei
05120	III	*Medicago falcata* L.	Hebei
13012	III	*Medicago falcata* L.	Jilin
13038, 13124 (nod^-)	III	*Medicago sativa* L.	Jilin
71001	III	*Medicago minima* L.	Shaanxi
71004	III	*Medicago sativa* L.	Shaanxi
71005	III	*Medicago lupulina* L.	Shaanxi
71121	III	*Medicago polymorpha* L.	Shaanxi
75006	III	*Medicago sativa* L.	Ningxia
75011	III	*Medicago falcata* L.	Ningxia
75035	III	*Medicago sativa* L.	Ningxia
81062	III	*Medicago archiducis-nicolai* Sirj	Qinghai
81007, 81099	III	*Medicago lupulina* L.	Qinghai
83027, 83052, **83062**, 83080, **83083**, **96071**	II	*Medicago sativa* L.	Xinjiang
83030	II	*Medicago falcata* L.	Xinjiang
83028, 83031, 83032	I	*Medicago falcata* L.	Xinjiang
83033	I	*Medicago* sp.	Xinjiang
83025, 83050, **83165**, 83166, 83167, **83177**, 83181, **83535**, 83536, **83537**, **96287**	I	*Medicago sativa* L.	Xinjiang

* Effective strains are in boldface and ineffective strains are underlined.

method was used to construct the dendrogram. The inoculated strains were included to compare with the isolates. Isolates sharing 100% similarity were identified as the same BOX type (strain).

Statistical analysis. All the nodulation and growth data were analysed by SAS for windows v8 (The SAS Institute Inc., Cary, NC, USA), and EXCEL 2002 (Microsoft corporation, 2001-2003). The variance in effectiveness of the strains was analysed with PROC ANOVA of SAS, in which Duncan's Multiple Range Test was applied and alpha = 0.01 was preferred to provide a 99% confidence limit.

The principal component analysis of SAS was used to evaluate affection of the different inoculants on the promotion the biomass accumulation and nodulation based on the alfalfa's dry weight of shoots (DWO), total number of nodules (TNN), and acetylene reducing activity (ARA). Each index had equal weights. Prin1 = 0.592792 DWO + 0.549094 TNN + 0.589147 ARA (cumulative proportion of eigenvalue difference = 84.72%); Prin2 = -0.356228 DWO + 0.834855 TNN – 0.419666 ARA (cumulative proportion of eigenvalue difference = 95.89%)

Nonlinear canonical correlation analysis of SPSS (SPSS 13.0 for Windows, SPSS Inc.) was applied to figure out the relations between the ecology type of strain's resource and the effective-

ness for the host plant. All the strains were divided into three ecogroups based on their geographical origins: (I) Inner Mongolia and North Xinjiang; (II) South Xinjiang; and (III) other areas including Hebei, Shanxi, Shaanxi, Gansu, Qinghai and Jilin (Lu, 1998). The DWO, TNN and ARA of different treatments were also divided into three grades with QUARTILE method (EXCEL), the high grade was more than 75% of total samples, which means high ability to promote the host plants, contrary, low grade was less than 25%, and the rest of samples (between 25 and 75%) was classed to intermediate grade.

Field experiment.

Location and soil characters. Field experiment was established in April 2005 at municipality of Horticulture Institute of Inner Mongolia (E 117.7′, N 40.8′), where annual average temperature is 4-6 °C, annual precipitation is 300 mm, and frost-free season is 120 days. The soil characteristics were the same as mentioned in "Screening step II" section. The field has no history for alfalfa growth, and was irrigated but not fertilised before planting.

Inoculants and seeds. To prepare the inoculants, rhizobial strains CCBAU 01199, CCBAU 01290, CCBAU 01314, CCBAU 13012 and CCBAU 83164 grown in TY broth were inoculated in tissue culture flask and incubated at 28 °C for 72 h. The cultures were centrifuged and the cell pellets were washed with sterilised saline solution (0.9% NaCl), homogenised using a mixer grinder, and the concentration adjusted to 10^{12} CFU/ml. The concentrated inoculums were diluted to 10^8 CFU/ml with tap water to make sure to inoculate about 10^5 CFU/seed. The alfalfa cultivars employed were Heigrazer, Vector and Aohan which were kindly offered by Dr. Zhao-Hai Zeng (College of Agronomy and Biotechnology, China Agricultural University). The seeds have germinating capacity up to 90-95%.

Field management and harvest. The seeds were sown in 54 blocks according to a randomized design of 3 (alfalfa cultivars) x 6 (5 rhizobial strains + 1 blank control) x 3 (repeats). The block area was 12 m^2 (2 x 6 m). Row planting was carried out with row spacing 20 cm, depth 3-5 cm, sowing rate 15 kg/hm^2. Seedbed inoculation method was used by inoculating the seeds with rhizobial suspension in the same time of sowing. After 50 days of sowing, shoots of alfalfa from each block were harvested randomly and dried at 60 °C for 48 h to constant weight. Dry weight of the shoots in each block was weighted and the data were analysed by SAS for windows v8 (The SAS Institute Inc.), and EXCEL 2002 (Microsoft corporation, 2001-2003).The variance effectiveness of strains was analysis with PROC ANOVA of SAS. Duncan's Multiple Range Test was also applied. A significance level of $P \leq 0.05$ was used for all statistical tests.

RESULTS

Strains characteristics

All the test strains formed colonies of 2-4 mm in diameter within 48 h on YMA, indicating that all of them are fast-growing rhizobial, despite of their different geological origins. All the strains are Gram-negative short rods. All the strains produced acid that was evidenced by the yellow colour in YMA supplied with BTB (Vincent, 1970).

Analysis of screening step I

Dry weight

The dry weights of shoots of alfalfa grown in vermiculite inoculated with each of the 96 rhizobial strains are showed in Fig. 1. In the statistic analysis, the dry weights of shoots inoculated with different strains were very different. Most of strains showed plant growth promoting, and the host biomass was increased significantly than control ($P < 0.0001$) by inoculation of 24 strains, respectively isolated from Inner Mongolia, Hebei and Xinjiang. The strains isolated from other areas such as Shanxi, Shaanxi, Gansu, Qinghai and Jilin showed lower effectiveness. In addition, 11 strains respectively isolated from Jilin, Shaanxi, Shanxi, Inner Mongolia and Xinjiang were not effective on the alfalfa cultivar involved in this study (Fig. 1 and Table 1). Some of them even caused yellow-green leaves, white freckle on surface of leaves, and rotten and brown roots in the inoculated plants.

Total number of nodules and acetylene reduction assay

The results are summarised in Fig 1. The total number of nodules and ARA showed a good correlation with each other and with the biomass production. Corresponding to the high dry weigh of shoots, the total number of nodules and ARA were also great. Additionally, the weaker ARA or smaller numbers of nodules were always related to the smaller biomass accumulation. Only one strain CCBAU 13124 from Jilin did not form any nodule on the host plants.

Nonlinear canonical correlation analysis

The results of this analysis, presented in Fig. 2, demonstrated that, over 99% confidence level, all the high and intermediate values of dry weights, nodule numbers, and acetylene reduction were closely associated with the rhizobial strains isolated from eco-region I, while the strains isolated from eco-regions II and III were closely related to the low values. These relationships indicated that the rhizobial strains were more effective with alfalfa grown in the same eco-region.

Principal component analysis

The principal component analysis of alfalfa biomass and nodulation showed that the test strains could be divided into three groups. Group 1 included 4 strains (CCBAU 01290, CCBAU 01199, CCBAU 01314, and CCBAU 83164) which were the most effective symbionts when all the data of biomass production, nodulation ability and ARA were considered. Group 2 included treatments with 23 isolates that had no significant difference with blank control. Group 3 covered the remaining strains that showed intermediate effectiveness.

Analysis of screen step II (nodule occupation)

Nodule occupancy

In this step, all the 4 strains in group 1, four randomly selected ineffective strains in group 2, and nine randomly selected intermediate effective strains in group 3 were used. To estimate the nodule occupancy, the BOX PCR fingerprinting of CCBAU 01290 is shown in Fig. 3 as an example. The nodule occupancy of the test strains is presented in Fig. 4.

Based upon the statistic analysis, inoculation of the 4 strains in group 1 (CCBAU 01290, CCBAU 01199, CCBAU 01314, CCBAU 83164) and 2 strains in group 2 (CCBAU 83025 and CCBAU 83535) caused significant increase of nodule numbers of the alfalfa plants compared with the blank control. However, only the 4 strains of group 1 and the group 2 strain CCBAU 83025 showed nodule occupancy more than 50%. These results demonstrated that the 4 most effective strains were also most competitive ones in the soil of Inner Mongolia. Furthermore, CCBAU 83025 was no effective in vermiculite, but rather competitive in soil (similar to CCBAU 01199 and CCBAU 01314), demonstrating that effectiveness and competitiveness were not always associated in a

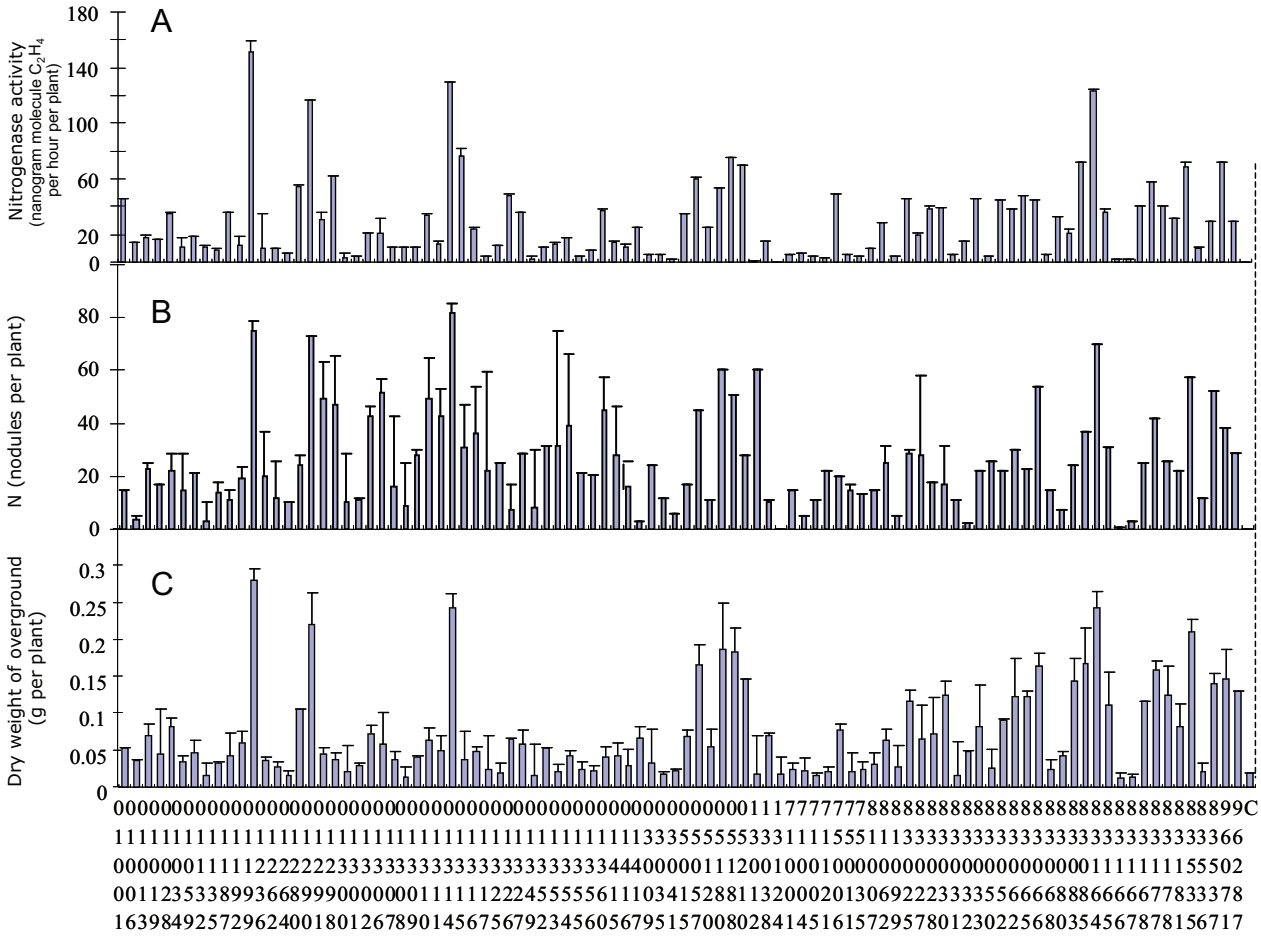

FIG. 1 - Effectiveness of alfalfa rhizobial obtained in the nodulation tests in vermiculite. A: Nitrogenase activity measured by acetylene reduction assay (nanogram molecular C_2H_4 per hour per plant). B: Total nodule numbers of alfalfa inoculated with different rhizobial strains. C: Dry matter of overground (DWO) of alfalfa inoculated with different rhizobial strains. All data were determined after 45 days after the inoculation. All the strains were from the Culture Collection, Beijing Agricultural University, China (CCBAU).

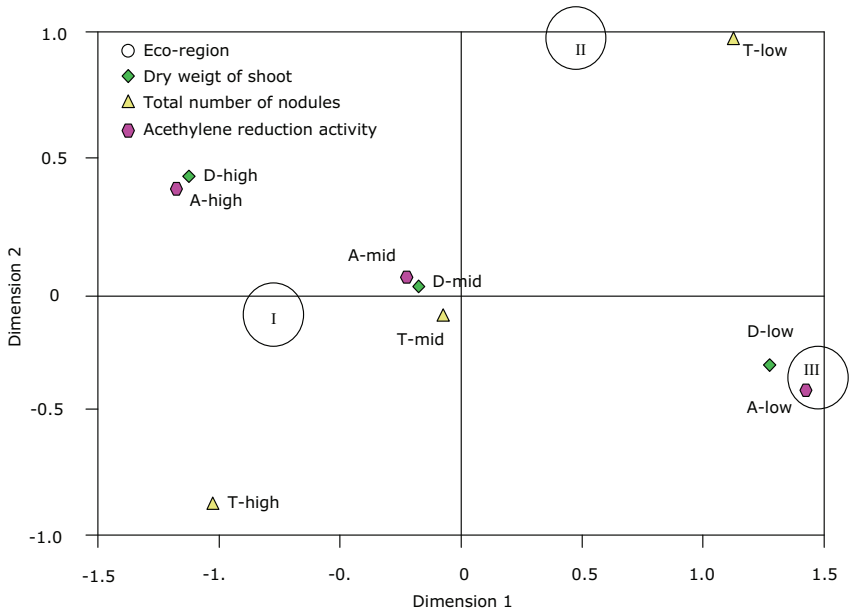

FIG. 2 - Nonlinear canonical correlation analysis among the eco-regions, the biomass accumulation, nodule numbers and nitrogenase activities. Confidence level was 99%. Optimal scaling level: ordinal. Three eco-regions (Inner Mongolia and North of Xijiang, South of Xinjiang, and other areas), three levels of increases (high = up to 75%, middle = 25-75%, and low = below 25%), in dry weight accumulation (D), total numbers of nodules (T), and acetylene reduction activity (A) were used as levels for each factor. Dimension 1 and Dimension 2 may have no real meaning.

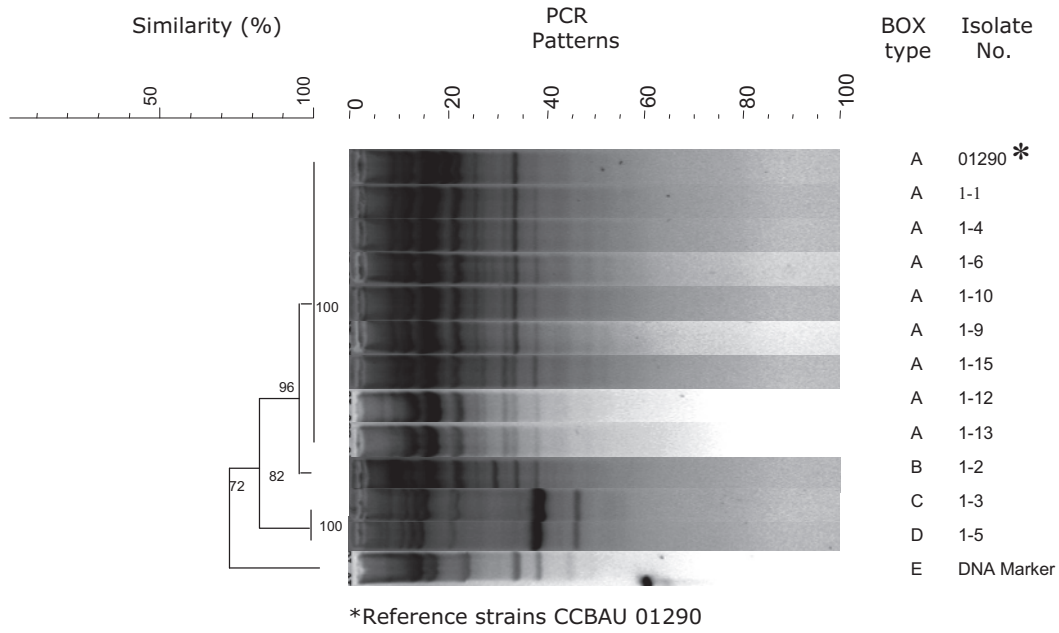

FIG. 3 - Example of BOX-PCR fingerprinting showing the comparison of nodule isolates and the inoculated strain (CCBAU 01290) that was used to estimate the nodule occupancy of the inoculant. Dice coefficient (optimisation: 5.00%, tolerance: 5.0%-10%) was calculated with the BOX PCR patterns and UPGMA (unweighted pair group method with arithmetic mean) method was used to construct the dendrogram. The isolates sharing 100% similarity were identified as the same BOX type (strain). Box type A was identified as the inoculated strain CCBAU 01290. BOX types B, C, and D represent indigenous rhizobia in the soil. BOX type E represent DNA marker (Molecular weight range from 2000 bp to 200 bp).

single strain. Most of the low and intermediately effective strains showed relatively low nodulation occupancy or competition in soil, especially the strain CCBAU 13012 that had the smallest occupation of 25%.

Biomass accumulation

In this test, the dry weights of alfalfa plants inoculated with test strains were compared. The results demonstrated clearly that only the four effective strains of the group 1 significantly enhanced the shoot weights of alfalfa in comparison with the blank control. Therefore, these four strains were both high efficient and high competitive rhizobial in association with alfalfa in soil of Inner Mongolia.

Field experiments

The data summarised in Table 2 demonstrated that the four strains of group 1 showed high competence and high efficiency in greenhouse experiments also significantly enhanced the biomass accumulation of alfalfa in fields, in comparison with the no inoculated control and with the inefficient strain CCBAU 13012.

DISCUSSION

The aim of inoculation of plants with microorganisms is to offer the plants some benefits, such as improve their growth or health. In the case of rhizobial, inoculation is for helping the legumes

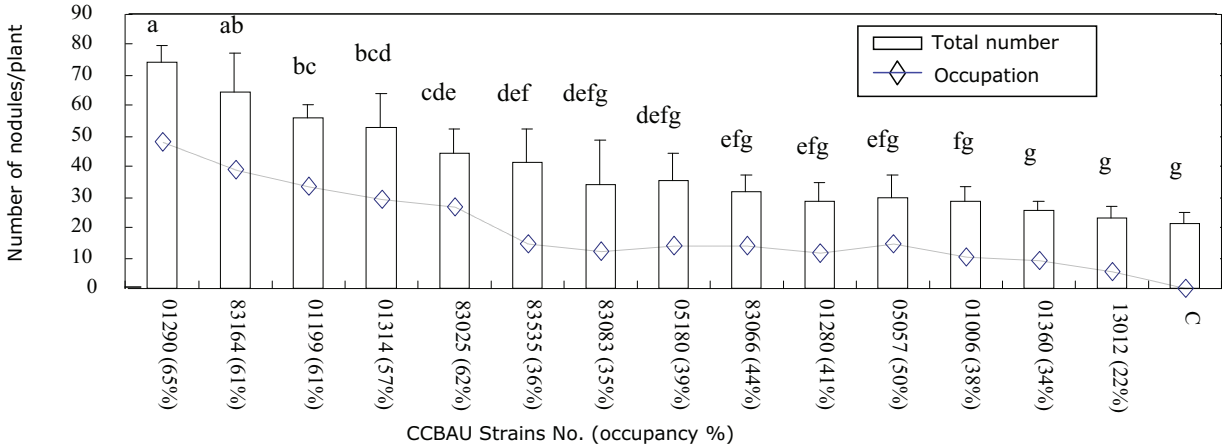

FIG. 4 - Results of competitive analysis of alfalfa rhizobial in natural soils. Total nodule numbers/occupation of inoculated strains, R-square = 0.812206 ($P < 0.0001$). Means marked with the same letters are not significantly different in the statistic analysis with Duncan's Multiple Range Test in ANOVA program of SAS software package.

TABLE 2 - Inoculation effects of rhizobial strains on alfalfa biomass accumulation

Inoculant	Average dry weight (g plant^{-1})*	Std. error
CCBAU 01199	4.114286[a]	0.774389
CCBAU 01290	4.156857[a]	0.768219
CCBAU 01314	3.525200[a]	0.739870
CCBAU 83164	3.566000[a]	0.622855
CCBAU 13012	3.171667[b]	0.475387
Blank control	3.030000[b]	0.267836

* Numbers marked with different letters were significantly different in statistic analysis.

to fix more nitrogen and hitherto to have a greater productivity. The success of inoculation of rhizobia depends on several factors, but the basal ones are the effectiveness related to the nodulation and nitrogen-fixation abilities and the competitiveness with indigenous bacterial populations (Thies *et al*., 1991). In general, screening of strains with high effectiveness and competitiveness as inoculants is performed with two-step strategy considering that good correlations between nodulation parameters and plant productivity are always obtained under N-deficient conditions (Somasegaran and Hoben, 1994), but some strains may be not good to be applied in natural soils due to the failure to compete with indigenous rhizobia (Streeter, 1994).

Aiming at selecting rhizobial inoculants for alfalfa grown in Inner Mongolia, a two-step protocol was carried out in this study to obtain the best strains and the results were confirmed with field experiments. Firstly, the effectiveness of all the strains was tested in greenhouse with the vermiculite as the nutrition-free media by combined analysis of dry weight of shoots, total number of nodules and activity of nitrogenase estimated by acetylene reducing activity. Secondly, the high effective strains selected in step I and other randomly chosen strains were employed for testing competition and effectiveness in natural soil collected from Inner Mongolia. The performance of competition of the inoculated strains with indigenous rhizobia in the soil was presented as nodule number and occupancy estimated with BOX-PCR fingerprinting, and the effectiveness of inoculated strains in soil was estimated from the increase of alfalfa biomass.

With this protocol, four most effective strains were obtained among the 96 test strains in the first step and they were also the most competitive and effective strains in the greenhouse experiments using natural soil of Inner Mongolia. All the four most effective strains were isolated from Inner Mongolia and North Xinjiang (eco-region I). This relationship between the nodulation parameters and geographic origin of the strains confirmed our previous observation, that the symbiosis between rhizobia and legumes is a result of interactions among the partners and environmental factors (Han *et al.,* 2005) and the demonstration of Galiana *et al*. (1990) that the most powerful strains were those isolated from nodules collected in the native range of the species. Recently, Lesueur *et al*. (2001) selected from 446 isolates 6 powerful strains nodulating with *Calliandra calothyrus*. These 6 strains were respectively isolated from Kenya, Costa Rica, and New-Caledonia, distributing in Africa and South America. However, this screen was performed only in sand and the soil conditions were not considered. Previously, we have observed that *Bradyrhizobium* sp. was predominant in nodules of common bean (*Phaseolus vulgaris*) grown in an acid soil (Han *et al*., 2005); *Sinorhizobium fredii* associated with soybean (Peng *et al*., 2002) and *S. meliloti* associated with alfalfa (Yan *et al*., 2000) in Xinjiang (alkaline-saline soils) were subpopulations different from those reported in other regions, where the genotypes of *Rhizobium leguminosarum* strains associated with faba bean (*Vicia faba*) showed clear biogeography in three eco-regions (Tian *et al.*, 2007). All the previous reports and the results in the present study demonstrated that the selection or introduction of rhizobial inoculants should consider the soil conditions or eco-regions.

Detection of target microorganism in environmental samples is a universal demand in studies on interrelations between microbe-microbe, microbe-materials and microbe-plant and animal, and one of the important models is rhizobia-legume interaction. With the development of molecular methods, immunology-based markers including fluorescent antibody technique (Schmidt *et al.*, 1968), enzyme linked immunosorbent assay and serological analysis (Berger *et al.*, 1979), protein-based markers including antibiotics, *GusA* (Jefferson *et al.*, 1987, Sessitsch *et al*., 1998)*, LacZ* and *LuxAB* (Cebolla *et al.*, 1991), and green fluorescent protein (Chalfie *et al.*, 1994), and DNA-based markers including RAPD (Random Amplified Polymorphism DNA) (Williams *et al.*, 1990), AFLP (Amplified frangment length Polymorphism) (Vos *et al.*, 1995) have been used. However, these methods could not satisfy the study due to background activity, no-repeatability, complexity and inscrutability interfered with exogenous genes. Therefore, some more sensitive and distinctive methods, such as BOX-A1R-PCR, ERIC-PCR, rep-PCR, DGGE and T-RFLP and so on were introduced into ecological studies (Gao *et al.*, 2001; Laguerre *et al.*, 2003; Thies *et al.*, 2004; Vachot-Griffin and Thies, 2005; Bogino *et al*., 2008). The latter techniques could detect the target bacteria based the original genetics, without any modified genes, and unforeseeable interference. In the present study, BOX-PCR functions very well in the differentiation of inoculated strains from the indigenous strains, although the alfalfa rhizobial have very similar phylogenies. Under the described procedure, we could obtain highly repeatable results. In addition, all the four best strains selected in the first step, estimated with principal component analysis, were exactly the great competitive ones in the second step and the best ones in field experiments. This relationship revealed the great value of principal component analysis and it might significantly enhance the efficiency of rhizobial inoculants selection.

To sum up, the two-step procedure employed in the present study was very effective in scanning large number of rhizobial strains for selection of inoculants. It was clear that the alfalfa rhizobial strains had very different capacities in nodulation, nitrogen-fixation and competition, and the most effective and competitive strains were all found among the test strains isolated from the same eco-regions of the target alfalfa cultivar, indicating the necessity of selection of inoculants according the eco-regions or ecotypes of the target plants. Based upon the results, we selected the 4 strains CCBAU 01199, CCBAU 01290, CCBAU

01314 and CCBAU 83164 as high effective and high competitive inoculants for alfalfa grown in eco-region I of China.

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