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S.S. Yang [a], R.A. Bellogín [b], A. Buendía [b], M. Camacho [c], M. Chen [d], T. Cubo [b], A. Daza [c], C.L. Díaz [e], M.R. Espuny [b], R. Gutiérrez [b], M. Harteveld [e], X.H. Li [a], M.C.C.P. Lyra [b], N. Madinabeitia [b], C. Medina [b], L. Miao [d], F.J. Ollero [b], M.M.A. Olsthoorn [f], D.N. Rodríguez [c], C. Santamaría [c], H.R.M. Schlaman [e], H.P. Spaink [e], F. Temprano [c], J.E. Thomas-Oates [f], A.A.N. Van Brussel [e], J.M. Vinardell [b], F. Xie [d], J. Yang [d], H.Y. Zhang [a], J. Zhen [a], J. Zhou [d], J.E. Ruiz-Sainz [b,*]

[a] *Department of Microbiology, China Agricultural University, 2 Yuan Ming Yuan West Road, 100094 Beijing, People's Republic of China*

[b] *Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apartado de Correos 1095, 41080 Sevilla, Spain*

[c] *Departamento de Suelos y Riego, CIFA Las Torres y Tomejil, Apartado de Correos Oficial 41200-Alcalá del Río, Sevilla, Spain*

[d] *Department of Microbiology, Huazhong Agricultural University, Shi Zi Shan Street, PO Box 430070, Wuhan, People's Republic of China*

[e] *Clusius Laboratory, Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, Leiden 2333AL, The Netherlands*

[f] *Department of Chemistry, Michael Barber Centre for Mass Spectrometry, UMIST, PO Box 88, Manchester, M60 1QD, UK*

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Abstract

Quantitative analyses of fast- and slow-growing soybean rhizobia populations in soils of four different provinces of China (Hubei, Shan Dong, Henan, and Xinjiang) have been carried out using the most probable number technique (MPN). All soils contained fast- (FSR) and slow-growing (SSR) soybean rhizobia. Asiatic and American soybean cultivars grown at acid, neutral and alkaline pH were used as trapping hosts for FSR and SSR strains. The estimated total indigenous soybean-rhizobia populations of the Xinjiang and Shan Dong soil samples greatly varied with the different soybean cultivars used. The soybean cultivar and the pH at which plants were grown also showed clear effects on the FSR/SSR rations isolated from nodules. Results of competition experiments between FSR and SSR strains supported the importance of the soybean cultivar and the pH on the outcome of competition for nodulation between FSR and SSR strains. In general, nodule occupancy by FSRs significantly increased at alkaline pH. Bacterial isolates from soybean cultivar Jing Dou 19 inoculated with Xinjiang soil nodulate cultivars Heinong 33 and Williams very poorly. Plasmid and lipopolysaccharide (LPS) profiles and PCR-RAPD analyses showed that cultivar Jing Dou

* Corresponding author. Tel.: +34-95-4557-121; fax: +34-95-4557-830.
E-mail address: rsainz@cica.es (J.E. Ruiz-Sainz).

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19 had trapped a diversity of FSR strains. Most of the isolates from soybean cultivar Heinong 33 inoculated with Xinjiang soil were able to nodulate Heinong 33 and Williams showed very similar, or identical, plasmid, LPS and PCR-RAPD profiles. All the strains isolated from Xinjiang province, regardless of the soybean cultivar used for trapping, showed similar nodulation factor (LCO) profiles as judged by thin layer chromatographic analyses. These results indicate that the existence of soybean rhizobia sub-populations showing marked cultivar specificity, can affect the estimation of total soybean rhizobia populations indigenous to the soil, and can also affect the diversity of soybean rhizobial strains isolated from soybean nodules.

Keywords: Soybean-rhizobia; MPN; Cultivar-specificity; Competitiveness

1. Introduction

Soybean cropping is extremely important in China where soybean seeds represent one of the major protein sources. In the last two decades it has become evident that soybean (*Glycine max*) can be nodulated by a wide variety of rhizobia (Van Berkum and Eardly, 1998). The main two groups of soybean microsymbionts are fast-growing bacteria (*Sinorhizobium fredii* and *Rhizobium xinjiangensis*) and slow-growing bacteria (*Bradyrhizobium japonicum*, *B. elkanii* and *B. liaoningense*). We will use the acronyms fast-growing soybean rhizobia (FSR) (1.5–4 h generation time), and slow-growing soybean rhizobia (SSR) (generation time over 6 h), to designate bacteria isolated from soybean nodules inoculated with the soil samples used in this work. Fast- and slow-growing bacteria that nodulate soybean will be collectively called soybean-rhizobia or soybean microsymbionts.

Soybean-rhizobia populations are common in the soil of soybean cropping areas of China, which is the geographical origin of soybeans. However, there are few reports on the distribution and relative predominance in Chinese soils of the two bacterial populations, their symbiotic nitrogen-fixation capacity as soybean inoculants and their competitive ability to nodulate soybeans (Devine, 1985; Dowdle and Bohlool, 1985, 1987; Buendía-Clavería et al., 1994). Earlier reports of competition experiments between FSR and SSR showed that in greenhouse conditions *Bradyrhizobium japonicum* strains usually outcompete *S. fredii* strains to nodulate Asiatic and American soybean cultivars (Dowdle and Bohlool, 1987; McLoughlin et al., 1985). In addition, the first set of *S. fredii* strains isolated from nodules of soybean plants growing in China (Keyser et al., 1982) form effective nitrogen-fixing symbioses with Asiatic soybean cultivars but are generally ineffective with American soybean cultivars (Keyser et al., 1982; Devine, 1985; Buendía-Clavería and Ruiz-Sainz, 1985). Later, the isolation of new *S. fredii* strains that are effective with American soybean cultivars were reported (Dowdle and Bohlool, 1985; Rodríguez-Navarro et al., 1996).

Since there is a pressing need in China to develop commercial soybean inoculants for agronomic use, we aimed to carry out quantitative analyses of FSR and SSR populations in four different regions of China in order to assess the chance of successful use of commercial soybean inoculants since the presence of large indigenous populations can represent a barrier to nodule occupancy by the inoculant strain. Due to the large diversity of soils in China where soybean inoculants could be used, we have also investigated the effect of pH on the balance of nodule occupancy between natural populations of FSR and SSR and also on their competition capacity to nodulate soybeans. *Rhizobium* indigenous populations are generally estimated by the Most Probable Number (MPN) technique, which requires inoculating legume plants with serial dilutions of the soil to be analysed. The number of plants that form nodules at each dilution level allows a statistical estimation of the number of bacterial cells that are able to nodulate that particular legume (Brockwell, 1963; Vincent, 1970). Although, it is known that the accuracy of this technique is limited (Thompson and Vincent, 1967), it is still widely used all over the world and computer programs have been developed for estimating populations from MPN data (Woomer et al., 1990).

The results presented were obtained during an INCO-DC project entitled "Improvement of symbiotic nitrogen fixation in Chinese soybean cropping areas" (ERBIC18CT970191). Here we show that the estimation of total indigenous soybean-rhizobia populations of a soil sample, the balance of nodule occupancy by fast- and slow-growers, and their competition capacity to nodulate soybeans can be greatly influenced by the particular soybean cultivar used and also by physical factors, such as the pH at which the plants are grown. We also show at the physiological and molecular levels that soil samples (such as that of Xinjiang region) can contain soybean-rhizobia indigenous sub-populations that show such a strong cultivar-specificity that the responses of soybean inoculation are determined by the particular soybean cultivar used.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions and media

Bacterial strains and plasmids used in this study are listed in Table 1. For growth of FSR strains, complete (TY) medium as described by Beringer (1974) or YMA medium as described by Vincent (1970) were used. SSR strains were grown in YMA medium. For LCO analyses, bacteria were grown in B^- medium as described by Van Brussel et al. (1977).

2.2. Nodulation tests

Nodulation tests were carried out on *Glycine max* cultivars Heinong 33, Linzhen, Jing Dou 19

Table 1
Bacterial strains and plasmids

Bacterial strains	Origin and relevant properties	Reference
Fast-growing soybean-rhizobia (FSR)		
S51	Isolate from nodules of soybean Williams inoculated with Honghu soil	This work
HW2, HWG32, HWG35	Isolates from nodules of soybean Heinong 33 inoculated with Shan Dong soil	This work
HH17	Isolate from nodules of soybean Heinong 33 inoculated with Henan soil	This work
WH11	Isolate from nodules of soybean Williams inoculated with Henan soil	This work
B10, B11, B12, B13, B14, B15, B16, B17, B47	Isolates from nodules of soybean Jing Dou 19 inoculated with Xinjiang soil	This work
B22, B24, B25, B27, B28, B29, B30, B31, B32, B33, B34, B42, B43, B44, B45	Isolates from nodules of soybean Heinong 33 inoculated with Xinjiang soil	This work
USDA 205 pR68.45	*S. fredii* USDA205 carrying plasmid pR68.45	This work
Slow-growing soybean-rhizobia (SSR)		
A8202	Isolate from nodules of soybean Jing Dou 19 inoculated with Hubei soil	This work
WWB20	Isolate from nodules of soybean Williams inoculated with Shan Dong soil	This work
HWB20	Isolate from nodules of soybean Heinong 33 inoculated with Shan Dong soil	This work
B55, B54	Isolates from nodules of soybean Williams inoculated with Xinjiang soil	This work
Plasmids		
pHN101	pIJ2925 carrying the *Vibrio fischeri luxAB* genes Ap^R	Li et al., 2000

(Asiatic cultivars), Williams, Bragg and Kobe (American cultivars). Soybean seeds were surface sterilised as described by Buendía-Clavería et al. (1994) or as described by Zhang et al. (1995). Germinated soybean seeds were transferred to Leonard jars containing sterilised vermiculite, supplemented with Fahraeus nutrient solution (Vincent, 1970) and inoculated with 1 ml of bacterial (or soil) suspension. The pH of the plant nutrient solution was adjusted to 6.0, 7.0, or 7.3 with 2.5 mM MES buffer [2-(*N*-morpholino)-ethanesulphonic acid] or 2.5 mM MOPS [3-(*N*-morpholino)-propanesulphonic acid] buffers, and to pH 8.0 with 2.5 mM MOPS or 2.5 mM Tris–HCl buffers. Inoculated soybean plants were grown for 5–7 weeks under greenhouse conditions. Plant tops were dried at 70 °C and weighed.

To quantify the indigenous soybean rhizobia population of soil samples, the MPN technique was carried out as described by Vincent (1970). Briefly, 10 g of soil samples were suspended in 90 ml sterile water and 10-fold serial dilutions of this initial soil suspension were carried out. Aliquots of 1 ml of each dilution level were used to inoculate soybean plants. Three (for Shan Dong and Henan provinces soil samples) and four (for Hubei and Xinjiang soils) replicates per dilution level were used for the estimation of the MPN. Inoculated soybean plants were grown for 5–7 weeks under greenhouse conditions, then scored for the presence or absence of nodules to determine the characteristic number. The appropriate statistical tables (Vincent, 1970), according to the dilution steps and the number of plants inoculated with each level of soil dilution, were used to estimate the most probable number of soybean-rhizobia present in the soil samples.

To determine nodule occupancy, soybean nodules were surface sterilised as described for soybean seeds. Surface-sterilised nodules were crushed on YMA media for single colony isolation. Those nodule isolates that produced visible colonies after 3 days in YMA media were assigned as FSR, those that need 5 days, or more, for producing visible colonies were considered SSR.

2.3. Competition for nodulation

Competition experiments between FSR and SSR were carried out on *Glycine max* cultivars Jing Dou 19, Heinong 33 and Williams. Bacteria were grown to mid-log phase and soybean plants were inoculated with 1 ml of a mixture of bacterial competitors in a 1:1 ratio. Plants were grown for 5 weeks under greenhouse conditions. Two different systems were used to determine the percentage of nodule occupancy by one of the bacterial competitors. In those competition experiments in which at least one of the competitors was a strain isolated from Xinjiang province ('B'-strains), intrinsic tetracycline resistance (only the slow-growing competitor can grow on YMA media supplemented with 1–5 μg tetracycline ml^{-1}) in combination with the speed of growth in YMA media were used to determine nodule occupancy. In the other competition experiments, a recombinant plasmid pHN110 was introduced into the fast-growing competitors. This plasmid carries the *luxAB* genes of *Vibrio fischeri* (Foram and Brown, 1988) and it is very stable in *Sinorhizobium meliloti* (Weinstein et al., 1992) and also in *S. fredii*. FSR strains carrying pHN101 emit light upon induction with *n*-decyl aldehyde. Soybean nodules were tested for the presence of plasmid pHN101 by 1 min exposure of X-film (Fuji) to soybean nodules treated with *n*-decyl aldehyde in a dark room.

2.4. Plasmid and LPS electrophoresis and RAPD analyses

Plasmid agarose electrophoresis was carried out as described by Espuny et al. (1987). Electrophoresis of bacterial lipopolysaccharides was carried out as described by Gil-Serrano et al. (1998). For the preparation of genomic DNA, rhizobial strains were grown in PA liquid medium (peptone 3 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}). Bacterial cells were pelleted by centrifugation, then washed three times with STE buffer (0.1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The pellet was resuspended in 0.4 ml lysis buffer (25 mM Tris–HCl, lysozyme 0.6 mg ml^{-1}, and RNase 20 μg ml^{-1}) and incubated at 37 °C for 30 min. Then,

250 μl 10% (w/v) sodium dodecyl sulphate (SDS) was added and the incubation was continued until the bacterial suspension became transparent. Proteins were removed by phenol and phenol–chloroform treatments. Finally, the DNA in the aqueous phase was precipitated with ethanol and dissolved in 100 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA concentration was estimated by agarose gel electrophoresis using a known concentration of lambda DNA.

For Random Amplified Polymorphic DNA (RAPD) analyses the following primers were used: P5, 5′-TCGGAGTGGC-3′; P14, 5′-CGGGAGACCC-3′and P17, 5′-GTTAGCGGCG-3′. RAPD was performed in a final volume of 25 μl of 1 × reaction buffer (10 mM Tris–HCl, 50 mM KCl) containing 20 ng DNA template, 2.5 mM $MgCl_2$, 50 ng of DNA primers, 0.5 U of Taq DNA-polymerase (Sangon Ltd, Shanghai), and 0.2 mM dNTPs. PCR amplification was carried out in a DNA thermal cycler (Perkin-Elmer Cetus, Beijing). The following profiles were used: 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 1 min; 1 cycle at 72 °C for 7 min. After PCR, 10 μl aliquots of products were electrophoresed in 1.5% (w/v) agarose gels for 2.5 h and visualised by ethidium bromide staining. The PCR Marker (Sangon Ltd, Shanghai) was included in all gels to estimate the size of the PCR-amplified fragments.

2.5. Isolation and analyses of LCO profiles

Bacterial cultures grown in liquid B^- medium to logarithmic-phase were diluted to an absorbance (OD_{620}) of 0.03 in 3 ml of B^- medium containing 0.6 μCi ^{14}C-D-glucosamine (specific activity of 50 mCi $mmol^{-1}$ Amersham, Buckinghamshire, UK). LCO production was induced by the addition of sterile seed exudate of *Glycine max* cv. Bragg and cultures were incubated at 16 °C for 20 h. Soybean seed exudate was obtained by shaking 50 seeds in 50 ml of 50% ethanol at pH 5.6 (using MES) for 18 h at 28 °C. The ethanol was evaporated in a rotorvap and the aqueous phase was passed over a C18 Sep-Pak cartridge (Waters) using the manufacturer's instructions. The seed exudate was sterilised by filtration over a 0.45 μm filter (Millipore) and its activity was tested over a range of dilutions (6–600 μl of a freshly prepared 1:1000 dilution was used in 3 ml bacterial culture for LCO labelling). In a pilot experiment it was found that 60 μl of this diluted seed exudate gave results comparable, with respect to the intensity of labelled spots, to when apigenin was used (data not shown). In the experiments described 600 μl of 1:1000 dilution of sterile seed exudate was used.

LCOs were extracted from the bacterial cultures using water-saturated *n*-butanol and analysed by thin layer chromatography (TLC) on silica-60 (Merck) and reverse phase-C18 (Merck) HP-TLC plates using *n*-butanol:ethanol:water (50:30:20 v/v/v) and 50% acetonitrile, respectively, as running solvents as described earlier (Olsthoorn et al., 2000). Radiolabelled spots were visualised using phospho-imaging (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. Quantitative analyses of soybean rhizobia populations in four regions of China

Serial dilutions of suspensions of soil samples from Wei Fang (Shan Dong province, Central-East China), Zheng Zhou (Henan province, Central-East China), Honghu (Hubei province, Central China) and Chang Ji counties (Xinjiang province, Western China) were collected and used as inoculants of Asiatic and American soybean cultivars. The total population of indigenous soybean-rhizobia in the four areas studied ranged from 10^4 to 10^6 bacteria per gram of soil (Table 2). The estimated levels of total indigenous soybean populations for Henan and Hubei soil samples were similar with both of the soybean cultivar used in the MPN experiment. However, the estimated soybean-rhizobia population of Xinjiang soil was clearly higher when soybean cultivar Jing Dou 19 was used than that estimated with cultivar Heinong 33. Similarly, the estimated soybean-rhizobia population of Shang Dong soil with Heinong 33 was higher than that estimated with Williams.

Table 2
Effect of pH and soybean cultivar on the isolation of soybean microsymbionts (FSR and SSR) from Chinese soil samples

Province of origin of the soil sample	Soybean cultivar	Estimated number of rhizobia ($\times 10^4$)[a]	Ratio FSR/SSR (%)[b]		
Henan			pH 6.0	pH 7.0	*pH 7.5*
	Heinong 33	4–50	A 73/27[a]	A 71/29[a]	A 73/27[a]
	Linzhen		A 64/36[a]	A 71/29[a]	A 71/29[a]
	Williams	2–30	A 60/40[a]	A 62/38[a]	A 65/35[a]
	Bragg		A 67/33[a]	A 56/44[a]	A 68/32[a]
Xinjiang			pH 6.0	*pH 7.3*	pH 8.0
	Jing Dou 19	4.2–51	100/0	100/0	100/0
	Heinong 33	0.06–0.8	100/0	99/1	100/0
	Williams		0/100	8/92	0/100
	Kobe		0/100	7/93	1/99
Hubei			pH 6.0	pH 7.0	*pH 8.0*
	Jing Dou 19	1–10	A 23/77[a]	B 56/44[a]	C 81/19[a]a
	Williams	1–10	A 27/73[a]	B 49/51[a]	C 90/10[a]
Shan Dong			pH 6.0	pH 7.0	*pH 7.5*
	Heinong 33	9–120	A 36/64[a]	A 29/71[a]	A 27/73[a]
	Linzhen		A 19/81[b]	A 21/79[a]	A 32/68[a]
	Williams	0.2–3.0	A 21/79[ab]	A 26/74[a]	A 29/71[a]
	Bragg		A 28/72[ab]	A 28/72[a]	A 36/64[a]

[a] The number of soybean-rhizobia (MNP) in each soil sample was estimated in soybean plants that were grown at the pH of the soil sample (indicated in underlined italics).

[b] Statistical analyses are among cultivars inoculated with the same soil sample. For each soil sample, numbers on the same line preceded by the same capital letter are not significantly different at the level $\alpha = 5\%$ using Fisher test for comparing proportions (effect of pH). Numbers in the same column followed by the same letter are not significantly different at the level $\alpha = 5\%$ using Fisher test for comparing proportions (effect of cultivar). Statistical analyses was not carried out for Xinjiang sample since the numbers are very different.

The relative capacity of soybean-nodule occupancy by FSR and SSR populations was estimated by determining the speed of growth of isolates from soybean nodules of plants inoculated at the lowest soil dilution, which was equivalent to adding 1 g of soil per Leonard jar. All soils contained FSR and SSR strains. The percentage of fast-growers recovered from soybean nodules was higher in three of the four investigated areas when soybean plants were grown at the pH value of the soil sample, although this prevalence of FSR strains on nodule occupancy of plants inoculated with Xinjiang soil was only observed for the Asiatic soybean cultivars (Table 2). In some cases, e.g. the Hubei soil sample, growing the soybean at acid pH (6.0) clearly increased the percentage of nodules formed by SSR strains. This effect of pH (favourable to SSR strains at acid pH, favourable to FSR strains at alkaline pH) was not observable with Shan Dong and Henan soils.

When plants were grown at the pH of the soil sample, the effect of the soybean cultivar on the ratio of FSR/SSR in nodule occupancy varied from very strong (Xinjiang soil) to no observable effect in the other three soils. The effect of the soybean cultivar on FSR/SSR ratios recovered from plants inoculated with Xinjiang soil sample was so dramatic that isolates from Jing Dou 19 nodules were all FSR while most of the isolates from Williams nodules were SSR.

3.2. Effect of pH and the soybean cultivar on competition between FSR and SSR strains

Results shown in Table 2 suggest that the soybean cultivar and the pH at which the plants are grown may affect the results of the competition

for nodulation of FSR and SSR indigenous populations and, as a result of this, the final outcome of nodule occupancy by these two bacterial groups. To investigate this possibility, 11 different pairs of competitors (FSR/SSR) were assayed for soybean nodulation at different pHs in different soybean cultivars. In six these pairs at pH 7.0–7.3, FSR strains were more competitive than SSR strains to nodulate the different soybean cultivars tested (Table 3). Results of competition experiments between pairs of competitors isolated from Shan Dong or Henan (Table 3, strains HW2, WH11, WWB20 and HWB20) clearly show that nodule occupancy by SSR competitors (WWB20 and HWB20) was increased at acid pH while FSR competitors (HW2 and WH11) were predominant at alkaline pH. This effect of pH was observed in both the Asiatic (Heinong 33) and the American (Williams) soybean cultivars. Results of competition experiments between pairs of competitors isolated from Xinjiang region (strains B42, B44, B47, B54 and B55) only showed this discriminatory effect of pH on nodule occupancy with the pairs B47/B54 (in cultivar Jing Dou 19) and B44/B55 (in cultivar Heinong). The outcome of competition experiments between the FSR-strain S51 (Hubei province) and the SSR-strain B54 (Xinjiang province) showed again a marked dependence on the pH at which soybean Jing Dou 19 plants were grown. The SSR-strain B54 strain was clearly outcompeted for nodulation of cultivar Jing Dou 19 by the FSR-strain HWG35 (Shan Dong province) at all pH values tested.

Earlier reports showed that, in general, *Bradyrhizobium japonicum* was more competitive than *Sinorhizobium fredii* to nodulate Asiatic and American soybean cultivars (Dowdle and Bohlool, 1987; McLoughlin et al., 1985). Since at pH 7.0–7.3, FSR-strains were more competitive in six out of eleven FSR/SSR pairs of competitors assayed, we tested new FSR/SSR pairs to confirm that the superior competition capacity of some FSR strains over SSR strains is not unusual. Table 4 shows that under the conditions tested (pH 7.0), FSR strains usually out-compete SSR strains to nodulate cultivar Jing Dou 19, the pair B45/B54 being the only exception.

3.3. Xinjiang soils contain a mixture of indigenous soybean-rhizobia sub-populations that show marked cultivar specificity

The estimated level of indigenous soybean-rhizobia populations in Xinjiang soil with soybean cultivar Jing Dou 19 was much higher than that estimated with cultivar Heinong 33 (Table 2). Further investigations with the bacterial isolates from Xinjiang soil were carried out to ascertain the reasons for these differences obtained using different soybean cultivars. For this purpose, 24 FSR isolates from Jing Dou 19 nodules were tested as inoculants of soybean cultivars Jing Dou 19, Heinong 33, and Williams. Results scored 30 days after inoculation showed that all the strains nodulated Jing Dou 19, but failed to nodulate Heinong 33 and Williams. When some of these strains were tested again with Heinong 33 and the plants were grown for 60 days, it was found that few, usually ineffective, nodules could be formed but soybean plants showed clear symptoms of nitrogen starvation. In contrast, isolates from Heinong 33 nodules were able to nodulate cultivars Jing Dou 19, Heinong 33 and Williams after 30 days of inoculation.

All the strains isolated from Heinong 33 show the same 4-plasmid profile (e.g. see Fig. 1B) while those isolates from Jing Dou 19 (Fig. 1A) show different plasmid profiles (1–3 plasmids), none of them similar to the typical plasmid profile of isolates from Heinong 33.

Differences in lipopolysaccharide (LPS) profiles between Xinjiang strains isolated from Heinong 33 nodules and those isolated from Jing Dou 19 nodules are also observed. Fig. 1D shows that LPS profiles of strains isolated from Heinong 33 (strains B27–B34) are similar. The LPS profile shown by these strains is very similar to that earlier reported for *S. fredii* HH103 (Gil-Serrano et al., 1998) consisting of a ladder with variable numbers of bands, which probably correspond to the complete LPS molecules (LPS-II region) and one or two faster migrating bands that could correspond to the lipid-A plus core and one, or zero, O-antigen subunits (LPS-I region). In contrast, LPS profiles of most of the strains isolated

from Jing Dou 19 (all but B16 and B47) do not have a ladder of bands in the LPS-II region but a large smearing spot, or poor staining in the LPS-I or LPS-II regions (Fig. 1C). The main difference between the two groups of isolates is that the standard technique for LPS profile visualisation gives repetitive profiles (bands) for isolates from Heinong 33 while it is variable from one experiment to another (in the intensity of the staining) for the isolates from Jing Dou 19. This variation in the efficiency of the technique indicates marked differences in the bacterial-surface components.

RAPD analyses using three different random primers of isolates from Heinong 33 and Jing Dou 19 showed again that strains isolated from Heinong 33 nodules were very similar, or identical, while different RAPD profiles were obtained from strains isolated from Jing Dou 19 nodules

Table 3
Competition experiments between FSR and SSR strains in different soybean cultivars grown at different pHs

Inoculant combination FSR/SSR at 1:1 ratio	Soybean cultivar	pH at which soybean plants were grown	Percentage of nodules formed by FSR strains[a]
S51/B54	Jing Dou 19	6.0	10.1 a
		7.0	65.9 b
		8.0	65.6 b
HWG35/B54	Jing Dou 19	6.0	86.5 a
		7.0	100 b
		8.0	96.7 b
B47/B54	Jing Dou 19	6.0	3.1 a
		7.0	10.0 ab
		8.0	16.9 b
B42/B54	Heinong 33	6.0	60 a
		7.3	65 a
		8.0	72 a
B42/B54	Williams	6.0	82 a
		7.3	86 a
		8.0	87 a
B44/B55	Heinong 33	6.0	58 a
		7.3	77 b
		8.0	80 b
B44/B55	Williams	6.0	79 a
		7.3	82 a
		8.0	83 a
HW2/WWB20	Heinong 33	6.5	23.6 a
		7.5	45.2 b
		8.5	70.5 c
HW2/WWB20	Williams	6.5	21.8 a
		7.5	40.0 b
		8.5	65.4 c
WH11/HWB20	Heinong 33	6.5	27.5 a
		7.5	36.3 a
		8.5	58.1 b
WH11/HWB20	Williams	6.5	32.7 a
		7.5	56.3 b
		8.5	78.1 c

Origin of the strains: Hubei province, S51. Xinjiang province, B42, B44, B47, B54, B55. Shan Dong province, HWG35, HW2, WWB20, HWB20. Henan province, WH11.

[a] Statistical analyses are among plants of the same cultivar inoculated with the same pair of competitors. For each cultivar and pair of competitors, numbers followed by the same letter are not significantly different at the level $\alpha = 5\%$ using Fisher test for comparing proportions.

Table 4
Nodule occupancy of soybean cultivar Jing Dou 19 with FSR/SSR pairs of competitors

Competitors[a]	SSR strains	
FSR strains	A8202 (Hubei)	B54 (Xinjiang)
S51 (Hubei)	97.8[b]	65.9
HH17 (Henan)	100	94.5
HHG32 (Henan)	97.7	87.5
HWG35 (Shan Dong)	100	100
B43 (Xinjiang)	100	92.3
B45 (Xinjiang)	84.1	10

[a] The ratio of competitors was 1:1, which corresponds to 10^9 cells per competitor.
[b] Numbers indicate the percentage of nodules occupied by *Sinorhizobium* strains.

(Fig. 2). In spite of all the differences observed in plasmid, LPS and RAPD profiles, strains isolated from the two soybean cultivars showed the same LCO profiles on silica (Fig. 1E) and reverse phase TLC analyses (Fig. 1F).

4. Discussion

Dowdle and Bohlool (1985) used a Chinese cultivar, Ai Jiao Zao, to trap the soybean microsymbionts present in soil samples from Honghu county (Hubei province). From these studies, it was concluded that high numbers of fast- and slow-growing soybean bacteria were present in the soil samples, although the latter were only detected when soybean plants were inoculated with highly diluted soil. Inoculation of diluted soil samples reduces the impact of bacterial competition for nodulation, probably due to the low number of soybean-nodulating bacteria applied to the root. Under these conditions, a broader diversity of rhizobial strains can be isolated (Muilenburg et al., 1996). Our results show that soil samples from four different regions of China contain both fast- and slow-growing soybean-rhizobia. Both bacterial populations were detected at the lowest soil dilution. Due to the large number of soybean-rhizobia populations in the samples analysed, bacteria probably compete for soybean nodule occupancy.

Since it is known that soybean rhizobia can show marked cultivar specificity (Keyser et al., 1982; Sadowsky et al., 1991), we have used different Asiatic and American soybean cultivars to estimate the balance of soybean nodule occupancy. Soybean cultivars inoculated with Xinjiang soil, and grown at the natural pH of the soil sample, showed significant differences in their ratio of FSR/SSR nodule occupancy. When soybean plants were grown under more acidic conditions (pH 6.0) new significant differences in the ratio of FSR/SSR nodule occupancy were scored among soybean cultivars that did not show differences in those plants grown at the natural pH of the soil sample. For instance, all cultivars inoculated with Henan soil gave a similar FSR/SSR ratio at pH 7.5 (natural) but the FSR/SSR ratio of cultivar Linzhen was significantly lower when soybean plants are grown at pH 6.0.

The effect of pH on the FSR/SSR balance of nodule occupancy is very clear with the soybean plants inoculated with Hubei soil sample, with FSRs significantly increased at alkaline pH. No significant differences due to pH are observed among plants of the same cultivar inoculated with Henan and Shan Dong soils, although there is a tendency to increase FSR nodule occupancy at alkaline pH. These results indicate that very complex strain/cultivar/pH interactions occur, making it difficult to predict whether or not the soybean cultivar and the pH will exert a strong influence on the balance of nodule occupancy by fast- and slow-growers. In addition, the differential effects of soybean cultivars and/or pH on the balance of fast/slow-growers recovered from nodules indicate that the soil samples contain very different soybean-rhizobia indigenous populations. These differences in nodulation ability of indigenous populations will have a fundamental impact on assessments of the possible successful use of soybean inoculants for a particular soybean cultivar in particular soil. It is also concluded that for those experiments aimed at isolating soybean rhizobia strains from soil samples, combining cultivars and pH should increase the possibility of isolating a wider range of soybean-nodulating rhizobia.

Variations in the nodule occupancy FSR/SSR ratio among different soybean cultivars or in the same cultivar at different pHs probably reflects the competitive ability of FSR and SSR strains to nodulate different soybean cultivars at different pHs. The few reports on competition for nodulation between *S. fredii* and *B. japonicum* showed that, under laboratory conditions, *B. japonicum* strains usually outcompete *S. fredii* strains for soybean nodulation (Dowdle and Bohlool, 1987; McLoughlin et al., 1985). Our results on the balance of nodule occupancy by FSR and SSR strains and the results of competition shown in Table 4 show that FSR strains can also be more competitive than SSR strains.

The results of 11 competition experiments be-

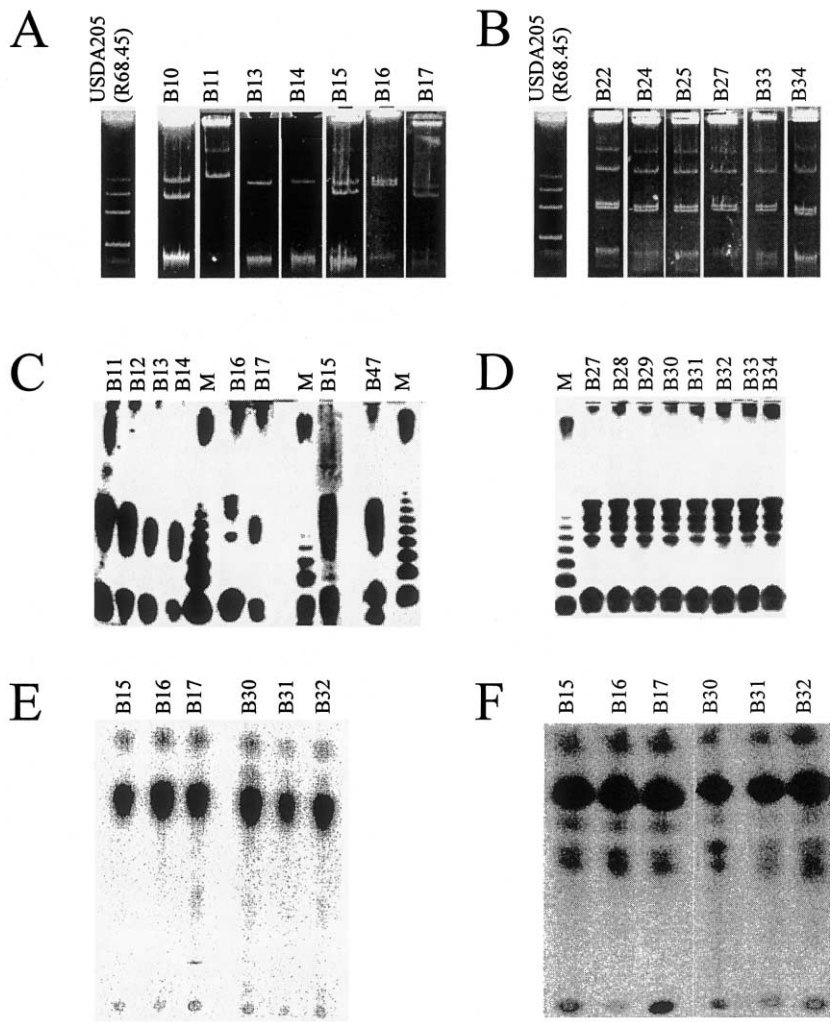

Fig. 1. Plasmid (A) and lipopolysaccharide (C) profiles of FSR strains isolated from soybean cultivar Jing Dou 19 inoculated with Xinjiang soil. Plasmid (B) and lipopolysaccharide (D) profiles of strains isolated from soybean cultivar Heinong 33 inoculated with Xinjiang soil. (E) Nodulation factor (LCO) profiles on silica TLC of strains isolated from cultivars Jing Dou 19 (B15, B16, B17) or Heinong 33 (B30, B31, B32). (F) Nodulation factor (LCO) profiles on reverse phase TLC of strains isolated from cultivars Jing Dou 19 or Heinong 33. LCO production by bacterial cultures was induced with soybean Bragg seed exudate. *S. fredii* USDA205 carrying plasmid pR68.45 (the smallest plasmid band) was used as a reference for plasmid profiles. (M) *Salmonella typhimurium* LPS (Sigma) was used as a reference for lipopolysaccharide profiles.

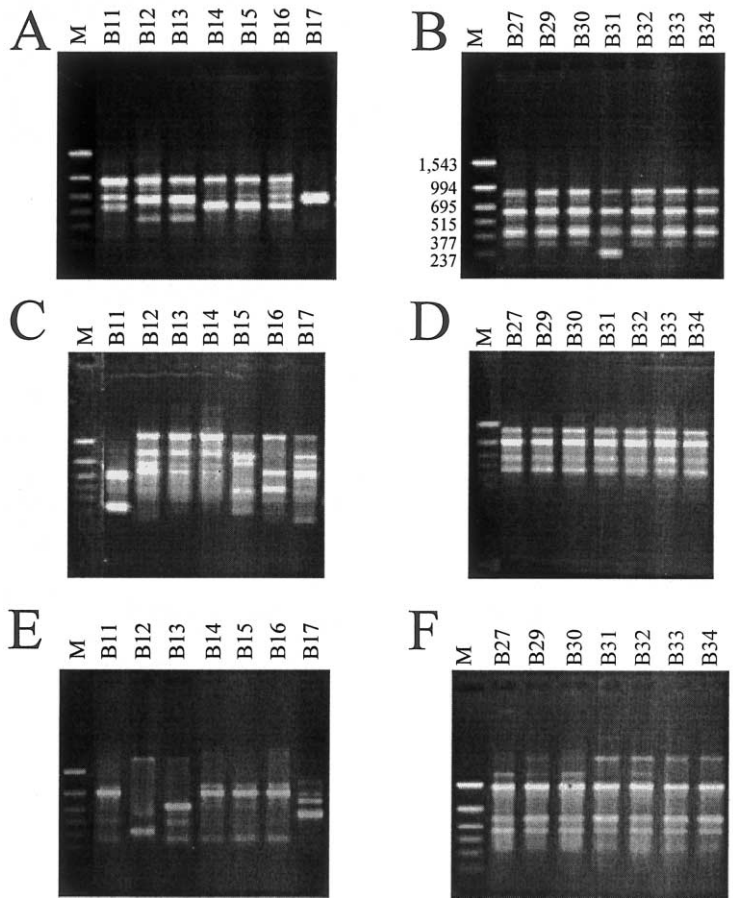

Fig. 2. RAPD profiles of FSR strains isolated from cultivars Jing Dou 19 (A, C, E) and Heinong 33 (B, D, F) inoculated with Xinjiang soil. Primers used: P5 (A, B), P14 (C, D) and P17 (E, F). M, molecular weight marker: 1543, 994, 695, 515, 377, 237 base pairs.

tween FSR and SSR strains at three different pH values (Table 3) supports the idea that, in general, alkaline pH increases soybean nodule occupancy by FSR strains. This effect was observed in seven FSR/SSR pairs of competitors in Asiatic and/or American soybean cultivars. In the other four FSR/SSR pairs, fast-growers were predominant at all pH values tested. Some of these FSR strains, such as S51 and HH17 appear to be highly effective with cultivar Jing Dou 19 in the greenhouse experiments (data not shown). To our knowledge, this is the first report describing FSR strains that are more competitive than SSR strains at acid pH. This finding opens the possibility of producing commercial FSR inoculants for acidic and alkaline soils, if highly competitive FSR strains finally prove also to be highly effective in any of the soybean cultivars tested.

The MPN technique is commonly used to estimate indigenous rhizobial populations in a soil sample (Brockwell, 1963; Crozat et al., 1982; Vincent, 1970). This information is of great importance for practical purposes, since high levels of indigenous populations will represent a serious barrier to successful nodulation by the inoculant. The results presented here clearly show that there are factors, such as the cultivar and the pH at which the plants are grown, that can affect the estimation of indigenous soybean-rhizobia when there are sub-populations showing a marked culti-

var specificity. A clear example is Xinjiang soil, in which an estimation of $0.06–0.8 \times 10^4$ bacteria per gram of soil is obtained when cultivar Heinong 33 is used in the MPN technique. These results would suggest that the number of soybean-nodulating bacteria indigenous to the soil is moderately low. Although even this moderately low number might be enough to prevent high nodule occupancy by the inoculant strain, this would not apparently face the disadvantage of competing for soybean nodulation with a large indigenous population. However, when soybean cultivar Jing Dou 19 is used as the trapping host the number of indigenous soybean rhizobia estimated by the MPN technique is much higher. Similar results were obtained with Shan Dong soil. Thus, rhizobial populations can be seriously under-estimated if an important fraction of the indigenous population is total or partially incompatible with the particular soybean cultivar used in the MPN assay. Since soybean cultivars are frequently changed in agricultural practice, inoculants applied to new soybean cultivars could be outcompeted by earlier undetected indigenous soybean rhizobia populations.

The presence of both fast- and slow-growing rhizobia in the Xinjiang soil sample was detected using different soybean cultivars. Thus it appears that the use of different soybean cultivars increases the chances of detecting bacterial sub-populations that could be partially or totally incompatible with specific cultivars or because they are not very abundant, that could be definitively lost if the soil sample is diluted. Most of the isolates from Heinong 33 nodules appear to be of the same strain, or group of closely related strains, as can be deduced from the similarities in their plasmid, LPS and PCR profiles. Although this strain(s) can nodulate Jing Dou 19, it was not found in the nodules of Jing Dou 19 plants inoculated with Xinjiang soil. Isolates from Jing Dou 19 nodules show clear differences in bacterial traits, showing that the cultivar is able to nodulate with a wide range of fast-growing strains and thus that the soil sample contains very different strains. These strains are very poor nodulators of cultivars Heinong 33 and Williams. A conclusion of these facts is that the degree of successful inoculation of soybeans under field conditions in those areas containing indigenous soybean rhizobia sub-populations showing strong cultivar specificity will be probably conditioned to the soybean cultivar used. PCR techniques aimed at estimating the total number of a determinate group of bacteria could be very complementary to the MPN technique, if specific primers are available.

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