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Inoculation Response of Alfalfa in Relation to Effectiveness and Competitiveness of Saudi Arabia Indigenous *Sinorhizobium meliloti* Strains

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Abstract. Nineteen *Sinorhizobium meliloti* strains from different field sites in Saudi Arabia were used to screen highly effective strains for alfalfa cultivar CAF 101 in a greenhouse study. Their symbiotic relationship and competitive ability were studied in the field. A randomized complete block design with three replicates and five seedlings per pot experiment was conducted at greenhouse conditions. Results showed that four *Sinorhizobium meliloti* strains were the most effective strains, as evidenced by increase in dry weights similarly with the field experiment. A field experiment showed that the inoculation of alfalfa with four strains of alfalfa rhizobia resulted in increases of 3.6-12.1% of dry matter production and 6.8-27.6% crude protein production. Obtained results showed that *S. meliloti* strain KSU 73 was an effective inoculant both in the greenhouse and in the field. The fluorescent antibody technique showed that the strain KSU 73 had high competitiveness in the field. It occupied 64% of nodules in alfalfa after 10 weeks of growth. In conclusion, a simple technique to select highly effective and competitive symbiotic strains specific to alfalfa was established.

Introduction

Alfalfa (*Medicago sativa* L.) is a deep-rooted, perennial legume capable of producing high yields of high-quality forage. Its excellent nutritional value makes this crop ideal for hay and silage. Alfalfa also has the ability to use atmospheric nitrogen (N_2) and deposit significant amounts of N in the soil during growth. A number of studies have shown that alfalfa cropping increases soil organic matter, improves soil structure, and builds up nitrogen (N) reserves in topsoil (Simpson, 1976; Holford, 1981; Peterson and Russelle, 1991). Alfalfa has a high forage yield and is resistant to harsh conditions. This forage crop occupies more than 30% of the completely cultivated area. The planting area of alfalfa has increased rapidly in the last few years, to reach about 103,000 hectares. Several previous studies have shown that the inoculation of legumes in general and alfalfa in particular with *Rhizobium* has the potential to increase production through improved biological N_2 fixation (Hardarson *et al.*, 1981; Wang *et al.*, 2002; Bolter *et al.*, 2005; Yu *et al.*, 2005; Zeng *et al.*, 2007). However, all strains of *Sinorhizobium meliloti* do not stimulate plant growth to a similar extent in a given alfalfa cultivar. A strain inducing superior performance in one cultivar may produce a suboptimal response in another. This interaction between the rhizobial strain and the host cultivar indicates that they must be matched carefully for optimum N_2 fixation (Gibson, 1962; Brockwell and Hely, 1966; Nutman, 1967; Gasser *et al.*, 1972; Tan and Tan, 1981; Zeng *et al.*, 2007). The screening of a highly effective strain for cultivar CAF 101 can significantly improve its production.

The identification of a highly effective strain for a given alfalfa cultivar does not mean that optimum symbiotic associations are established at field conditions. In soil, the indigenous rhizobia reduce the effectiveness of the inoculated strains by competing for the nodulation of the host plant. Therefore, one desirable characteristic of the inoculated legume strain is its ability to compete with indigenous rhizobia for infection sites (competitiveness) (Brockwell *et al.*, 1968; Leach, 1968; Brockwell *et al.*, 1982; Zeng *et al.*, 2007). Because the competitive ability of the inoculated strains and their effectiveness appear to be independent traits in

Sinorhizobium meliloti (Amarger, 1981; Singleton and Tavares, 1986), there is a need to develop simple methods for identifying the inoculated rhizobial strains recovered from nodules to evaluate their competitiveness. Such methods should not only be reliable and reasonably specific but also simple enough to be applied to a large number of strains. Cited literatures stated that fluorescent antibody technique (FA) is a useful adjunct to serological methods for the rapid identification and characterization of strains in the nodules, but the main promise of FA in *Rhizobium* ecology lies in the study of events prior to nodulation (Bohlool and Schmidt, 1980; Abdel-Aziz and Al-Barakah, 2003).

Interaction between alfalfa cultivars and rhizobial strains has been extensively studied. But little information is available on the screening of highly effective strains for an alfalfa cultivar or on the evaluation of competitiveness of rhizobial strains in association with a certain cultivar in the field. Therefore, studies on the screening of highly effective strains and on nodule occupancy by inoculated strains in the field are necessary to judge whether a rhizobial strain is suitable for inoculant production. The objectives of the present study were (1) to screen highly indigenous effective strains for alfalfa cultivar at greenhouse conditions and to test their effect in the field under Saudi Arabian conditions, and (2) to apply fluorescent antibody technique to identify their competitive ability in the field.

Materials and Methods

Bacterial strains

Nineteen *Sinorhizobium meliloti* strains were isolated from root nodules of *Medicago sativa* grown in seven provinces in the temperate regions of Saudi Arabia. All the strains were grown on yeast mannitol agar (YMA) medium (Vincent, 1970) at 28°C and were stored in 20% glycerol at -70°C. Single colonies were chosen for further culturing.

Greenhouse experiment

The alfalfa cultivar used in this experiment was *Medicago sativa* cv. CAF 101. Alfalfa seeds were germinated aseptically on Petri dishes containing agar. After germination, seedlings with 1-2 cm of radical were planted in plastic pots 15 cm in diameter filled with soil obtained from Al-Kharj County. A randomized complete block design with three replicates was used, and five seedlings were planted per pot. The inoculation of the seedlings with each rhizobial strain was done immediately after planting. Each germinated seed received approximately 10^8 viable rhizobial cells. To prevent cross-contamination among treatments, a thin sheet of clear plastic was wrapped to cover the top surface of the pot, leaving small holes for the seedlings to grow through. The experiment was conducted in a greenhouse with temperatures of 23 ± 5°C in the day and 13 ± 3°C in the night, with supplemental fluorescent lighting providing a 12-hour photoperiod. The seedlings were grown for 10 weeks after inoculation. At harvest, five seedlings from each pot were taken. Seedlings dry weight was determined after drying at 60°C to constant weight. Nitrogen content was determined by micro-Kjeldahl.

Field experiment

The field experiment was conducted in 2006 at Al-Kharj County, Riyadh, Saudi Arabia. The soil texture is sandy loam; soil organic matter and total soil N averaged 3.9 and 0.31 g kg^{-1}, respectively. Total digestible P and K were 7.2 and 43.5 mg kg^{-1}, respectively. The average pH of the soil was 8.2. A randomized complete block design with three replicates was arranged in the field. Basal application rates before seeding were 40 kg K ha^{-1} (as K_2SO_4) and 20 kg P ha^{-1} (as Ca $(H_2P0_4)_2$-$2H_20$). Plots were 7 m long and 3 m wide. The alfalfa cultivar CAF 101 was sown 200 mm apart in rows and seeding rates of alfalfa was 30 kg ha^{-1}. Five treatments were as follows:

1. Un-inoculated and fertilized with the Ministry of Agriculture recommendation.
2. Inoculated with Strain KSU 73.
3. Inoculated with Strain KSU 121.
4. Inoculated with Strain KSU 176.
5. Inoculated with Strain KSU 188.

Growth of cultures and seed coating

Four strains were selected on the basis of the results of experiment in the greenhouse. These strains were grown on YMA medium in incubator shaker at 28°C to early stationary phase. Then the culture was washed twice using sterile water, by means of suspension and centrifugation, before the planting date. The washing was to remove the YMA medium components that may affect the nodulation of the plants. After the second wash, the cells were suspended in sterile water with a final density of 2 x 10^8 cells mL-^{1}. The inoculant was applied to the seed at the rate of 50 ml kg^{-1} seeds, so that the inoculation rate of 10^7 cells per seed was obtained. Un-inoculated control was prepared with sterile water. The seeds were planted in the field in November 2006, first by sowing the un-inoculated control plot and then the inoculated treatment plots. Plots were irrigated as required and weeded by hand.

Above ground dry matter (DM) production (yield) was measured by clipping two 1-m^2 quadrats per plot. Protein content was also calculated. Alfalfa was harvested three times in 2007 after 70, 105, and 140 days from planting. At each harvest, all roots from a 1-m^2 section of the row were sampled by digging to a depth of 20 cm. After excavation, the roots were washed and the nodules from all roots or from a representative number of roots were excised and weighted after dried at 60°C.

Nodule serotyping

Strain specific fluorescent antibodies (FAs) were used for nodule serotyping. Gelatin-Rhoda mine conjugate (Somasegaran and Hoben, 1985) was used to control non-specific staining and auto fluorescence. Fifty nodules from each treatment were carefully washed and crushed in sterilized water. The slides were air dried and heat fixed. With a Pasteur pipette, a drop of gelatin-Rhoda mine conjugate was placed on the smears. Before the Rhoda mine gel was dried, one drop of FA stain was added. Stained nodule smears were examined with a Zeiss universal microscope equipped for epifluorescence and phase contrast. A strong positive reaction was indicated by brilliant yellow green fluorescence of the smear on a dark purple background. No cells would be visible if the specific strain was not present on the smear. The presence of more than one serogroup per nodule was detected by using the dual lighting system of reflected fluorescent and transmitted phase-contrast light. The switching from phase-contrast to fluorescent light, clearly shows the presence of one or more than one strain in the same nodule. The dominance of one strain over the other within the same nodule was based on the ratio between the stained and non stained cells with each FA. Nodule smears with 5% or more of non-fluorescing cells in the presence of FA-positive cells were considered evidence of a mixed infection.

Statistical analysis of data

Yield data from each field harvest and the dry weight of plants in the greenhouse were analyzed statistically using the general linear model procedure of SAS (SAS Inst., 1996). Mean comparisons were made with an F-protected LSD at $P < 0.05$.

Results

Greenhouse experiment

Significant differences were found between the alfalfa plants inoculated with the different *Sinorhizobium meliloti* strains (Table 1). Of the 19 strains, four strains were the most effective strains, as shown by the maximum values recorded for plant dry weight (Table 1). KSU 73 was the most effective strain, whereas KSU 69 was the least effective one because the plants inoculated with this strain grew as poorly as the control. The plants inoculated with the other strains showed higher dry weight compared with the un-inoculated controls. Plant nitrogen content was also determined in this experiment. By comparing the plant dry weight, and nitrogen content, the most effective four strains were selected for the field study.

Field experiment

All of the dry weight of nodules per plant, forage dry matter production, and crude protein content of the alfalfa plants increased, when inoculated with the selected four strains (Table 2). Nodules dry weight per plant was significantly higher in the inoculated plants than those recorded in the uninoculated N-fertilized treatment. This finding clearly indicates that the infectivity of the tested strains were greatly higher than those of native *S. meliloti* strains. Results also showed that, strain KSU 73 was found to be superior in nodules formation as compared with the other introduced strains.

Results showed that in all treatments, the alfalfa yield varied in the successive cuttings. The highest yields were that of the third cut followed by the second one. In the first cut the plants were still comparatively thin, since they recorded low dry matter yields. Data in Table 2 show that the inoculation of alfalfa seeds with the introduced four strains induced significant increase in dry matter yield as compared with the un-inoculated N-fertilized control. These results proved that we could reach the same or more alfalfa dry matter yield by rhizobial inoculation without any need to add N-fertilizers. Significant differences in dry matter yields among different inoculation treatments were observed. In this respect, strain KSU 73 was superior, since it recorded the highest dry matter yield followed by strains KSU 176, 188, and 121 in respective order.

As mentioned before (Table 2) the highest yield was that of the second and third cuttings, and the lowest one was that of the first cut. It is not surprising, therefore, that the maximum amount of N-fixed and subsequent crude protein yield was found in the second and third cuttings in all treatments. Thus, the rate of N_2-fixation parallels to the vigors of growth. Although, the un-inoculated N-fertilized alfalfa plants formed a considerable amount of nodules, these nodules fixed the lowest amount of atmospheric nitrogen in the three cuttings as

Table 1. Dry weight and N-content of alfalfa plants inoculated with different *Sinorhizobium meliloti* strains isolated from different geographical origins in a greenhouse experiment

Strain	Origin	Dry Weight (g / plant)	N-content (mg / plant)
Uninoculated	-	0.287	10.045
KSU16	Qassim	0.520	14.815
KSU 30	Qassim	0.536	15.431
KSU 41	Qassim	0.488	16.104
KSU 69	Qassim	0.409	14.142
KSU 73	Hail	0.813	24.024
KSU 75	Hail	0.509	14.013
KSU 76	Hail	0.515	14.827
KSU 100	Hail	0.686	18.536
KSU 121	Al-Kharj	0.726	20.571
KSU 140	Wadi Aldawaser	0.609	15.931
KSU 153	Wadi Aldawaser	0.644	14.393
KSU 154	Wadi Aldawaser	0.581	16.204
KSU 156	Wadi Aldawaser	0.681	17.686
KSU 167	Al-Hassa	0.662	17.578
KSU 175	Al-Hassa	0.421	14.549
KSU 176	Al-Hassa	0.769	22.371
KSU 185	Al-Madina	0.509	17.403
KSU 188	Al-Madina	0.748	20.949
KSU 229	Tabouk	0.555	16.317
L.S.D at 0.5 %		0.112	3.185

Table 2. Nodules dry weight, dry matter yield and crude protein of alfalfa inoculated with different *S. meliloti* strains

Treatments	Nodules Dry Weight (mg / plant)			Dry Matter Yield (Kg / ha)				Crude Protein Yield (Kg / ha)			
	Cuttings			Cuttings				Cuttings			
	1st	2nd	3rd	1st	2nd	3rd	Total	1st	2nd	3rd	Total
Un-inoculated and N-fertilized	9.8	18.1	21.8	1897	2126	2309	6332	391.3	438.5	476.2	1305.9
Inoculated with KSU 73	15.4	23.6	33.2	2186	2365	2548	7099	513.2	555.2	598.1	1666.5
Inoculated with KSU 121	11.2	19.1	26.3	2017	2192	2353	6562	428.6	465.8	500.1	1394.4
Inoculated with KSU 176	13.2	22.3	29.1	2088	2297	2424	6809	469.8	516.8	545.4	1532.1
Inoculated with KSU 188	11.2	21.2	27.5	2064	2243	2368	6675	451.5	490.7	518.0	1460.2
L. S. D. (0.05)	1.1	3.1	4.8	118.4	127.7	160.3	406.8	32.8	37.6	43.2	166.3

compared with the inoculated treatments. Data also show that the inoculation with effective *S. meliloti* strains exerted higher amount of N_2-fixed as compared with the un-inoculated N-fertilized control. This means that the introduced *S. meliloti* strains can fix a considerable amount of atmospheric nitrogen equal or more than the added nitrogen. The results also showed that the inoculated treatment with strain KSU 73 exhibited the highest amount crude protein yield as compared with the values recorded in the other inoculated treatments.

Nodule occupancy estimated by FA technique

Results of competition for nodulation between indigenous and four introduced *S. meliloti* strains on alfalfa root system are presented in Table 3. As shown, in the un-inoculated treatment, the percentage of nodule occupancy with native rhizobia was 81%. At the same time, 7, 3, 6, and 3% of the nodules were formed by the introduced *S. meliloti* strains KSU 73,

Table 3. Competition for nodulation between indigenous and four introduced *S. meliloti* strains on alfalfa root system

Treatments	Percentage of Nodule Occupancy by Strains No.				
	KSU 73	KSU 121	KSU 176	KSU 188	Native Rhizobia
Un-inoculated N-fertilized control	7	3	6	3	81
Inoculated with strain KSU 73	64	-	-	-	36
Inoculated with strain KSU 121	-	43	-	-	57
Inoculated with strain KSU 176	-	-	56	-	44
Inoculated with strain KSU 188	-	-	-	48	52

121, 176, and 188, respectively. These results can be explained by the fact that these introduced strains were locally isolated from Saudi Arabian soils and it usually successfully nodulate alfalfa plants.

Data also show that the highest percentage of nodule occupancy in the inoculated treatments was formed by *S. meliloti* strain KSU 73, as it was estimated by 64%. On the other hand, the strains KSU 121, 176, and 188 occupied 43, 56, and 48% of the total formed nodules in respective order. These results clearly indicate that the introduced *S. meliloti* strain KSU 73 was considered to be the most competitor strain to both the other introduced inoculant strains and native rhizobia. Therefore, the higher the competition between introduced strains and native rhizobia, the higher the yield production and crude protein content of alfalfa and vice versa.

Discussion

Knowledge of the symbiotic characteristics of the indigenous population is required to predict the outcome of inoculation (Brockwell *et al.*, 1988). The symbiotic effectiveness of indigenous isolates from alfalfa legume is presented in Table 1. Plant dry matter production was strongly correlated with total plant nitrogen; therefore, the biomass production was used as criterion strain effectiveness in N_2 fixation.

Many new alfalfa varieties have been introduced to Saudi Arabia from other countries. Considering that the genetic background of a plant is a determinative biological factor to select the rhizobia associated with each variety, screening for highly effective symbiotic strains with improved competitive ability is very important. Chen *et al.* (2002) conducted experiments in greenhouses to screen highly effective symbiotic strains against four alfalfa cultivars grown in vermiculite. Although the dry weight of alfalfa inoculated with rhizobia significantly increased in the greenhouse, the hay yield did not increase in the field compared with the un-inoculated plants. These results indicated that some rhizobial strains may be very effective in nitrogen fixation, but they could not compete with the indigenous rhizobia in the field. In this experiment, four highly effective strains were selected for the CAF 101 cultivar by using non-surface sterilized seeds and growing the plants in non-sterilized soils collected from fields where the "CAF101" alfalfa was planted. In these cases, the inoculated rhizobial strains were competing with the indigenous rhizobia when the experiment began.

The selection of strains with improved competitive ability is always one of the most important criteria in the development of improved commercial legume inoculants. The competitive ability of the selected strains can be determined by testing the nodule occupancy in the field. Different methods have traditionally been used in ecological studies of *Rhizobium* spp. in soil and in association with plants (Bohlool and Schmidt, 1980; Morley and Jones, 1980; Noel and Brill, 1980; Bushby, 1981). The fluorescent antibody (FA) technique was applied to test competition in the nodulation ability of alfalfa Sinorhizobia. The results of this study indicated that the FA technique was a useful tool to estimate the competitive capability of an inoculant by determining its nodule occupancy.

An important objective in legumes inoculation research is to select highly effective strains of rhizobia for a particular host plant. These strains must also be able to establish themselves in the rhizosphere and compete successfully for nodule sites against the indigenous soil rhizobia, which often include ineffective strains. The introduced strain has to compete, not only with other rhizobia, but also with the soil population, for substrates and space in locations already occupied.

Most inoculated legume seeds are sown into soil containing indigenous rhizobial populations that are often inferior in effectiveness compared with the inoculated strain and compete with it for nodulation of the host plant. Bosworth *et al.* (1994) inoculated alfalfa with *Sinorhizobium meliloti* strain harboring an extra copy of *dctABD* at Marshfield, where the indigenous *S. meliloti* population was 10^4 cells g^{-1} dry soil. Thirty days after planting, nodule occupancy by the inoculated strain was 18.2%, and the strain was permitted limited commercialization by the Environmental Protection Agency in 1997. In our experiment, the indigenous *S. meliloti* population was 6 x 10^4 cells g^{-1} dry soil. Ten weeks after the inoculation, the nodule occupancy of strain KSU 73 in this experiment was 64%. Results indicated that strain KSU 73 has high competitiveness and persistence and could be a suitable inoculant.

Results on symbiotic effectiveness confirmed observations of the previous studies (Jebara *et al.*, 2001; Mihaela Bradic *et al.*, 2003; Al-Asmary, 2007) that isolates of *S. meliloti* vary in their N_2 fixation ability. In the present study, the majority of the field isolates were low in N_2 fixation effectiveness. It is, therefore, apparent that the inoculation of alfalfa plants with selected highly effective and competitive rhizobial strains is needed. Differences in N_2 fixation capacity within the tested isolates might be attributed to ecological factors in the ecosystem from which the strains were isolated or may be related to the loss of important genetic information related to symbiotic performance of the isolates due to long exposure to the environmental stress in the soil. Such variability was reported previously in field population of *R. leguminosarum* bv. *trifolii* (Valdivia *et al.*, 1988), *Sinorhizobium meliloti* (Jebara *et al.*, 2001; Mihaela Bradic *et al.*, 2003; Al-Asmary, 2007; Zeng *et al.*, 2007).

Conclusions

The greenhouse screening system used, including non-surface sterilized seeds and growing the plants in natural soils, was well correlated with the results in field trials and it could be a model to screen highly-effective symbiotic strains for legumes. Strain KSU 73 was a suitable candidate as inoculant for alfalfa cultivar CAF 101. Although indigenous rhizobia existed in large numbers in the fields, inoculation with a suitable strain could enhance the biomass production of the inoculated host legume and the co-cultivated plants. It could be also concluded that conditions in the Saudi Arabian soils favor active symbiotic N_2-fixation by effective introduced *S. meliloti* strains, and this proved the importance of alfalfa in maintaining soil fertility. Since this study was conducted only for one year and only the FA method was applied to test the nodule occupancy, further studies are needed to compare different methods to test the rhizobial inoculant and its dynamics in the field.

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استجابة البرسيم الحجازي للتلقيح وعلاقته بالفاعلية والقدرة التنافسية لسلالات الريزوبيا المتوطنة بالمملكة العربية السعودية

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ملخص البحث. تم استخدام ١٩ سلالة من سلالات ريزوبيا البرسيم الحجازي تم عزلها من مواقع حقلية مختلفة بالمملكة العربية السعودية، وذلك لتحديد السلالات ذات الفاعلية العالية في تثبيت النيتروجين الجوي بالتكافل مع صنف البرسيم الحجازي (كاف ١٠١) في تجربة صوبة زجاجية، وكذلك لدراسة علاقتها التكافلية وقدرتها التنافسية في تجربة حقلية. وقد أوضحت تجربة الصوبة أن أربع سلالات من سلالات ريزوبيا البرسيم الحجازي المستخدمة كانت الأكثر فاعلية حيث كانت الأعلى في الأوزان الجافة والبروتين الخام، حيث أظهرت التجربة الحقلية أن تلقيح البرسيم الحجازي بالسلالات الأربعة المختارة حققت زيادة من ٣,٦-١٢,١٪ في إنتاج المادة الجافة، ومن ٦,٨-٢٧,٦٪ في إنتاج البروتين الخام. وقد أوضحت النتائج أن سلالة ريزوبيا البرسيم الحجازي رقم ٧٣ (KSU 73) تعتبر لقاحاً ذا فاعلية عالية في كل من تجربتي الصوبة والحقل. ومن ناحية أخرى أظهرت تقنية الأجسام المضادة الفلورسنتية أن السلالة رقم ٧٣ (KSU 73) حققت قدرة تنافسية عالية في الحقل حيث احتلت ٦٤٪ من العقد الجذرية على البرسيم الحجازي بعد مرور ١٠ أسابيع من بداية النمو. ومما سبق نستنتج أنه باستخدام تقنية بسيطة يمكن اختيار سلالات متخصصة للبرسيم الحجازي ذات فاعلية وقدرة تنافسية عالية يمكن أن تؤدي إلى زيادة الإنتاجية.