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COMPETITIVENESS OF INTRODUCED *RHIZOBIUM* STRAINS FOR NODULATION IN FODDER LEGUMES

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Abstract

Rhizobial strains were isolated from the root nodules of *Medicago sativa* (alfalfa) and *Trifolium subterraneum* (clover) plants. These strains were tested for their growth rate, morphological characteristics and utilization of different carbon sources. Four out of six strains isolated from the alfalfa plants re-nodulated the host plant confirming them as the strains of *Rhizobium meliloti*. Similarly five out of eight strains isolated from clover plants caused infection on clover corroborated as *Rhizobium leguminosarum* bv. *trifolii* strains. The nitrogen fixing ability of these strains ranged from 266 - 673 n moles of C_2H_4 produced $h^{-1}g^{-1}$ nodule dry weight. The competitive ability of inoculated strains with indigenous population was studied with two local soils selected on the basis of their cropping history in a pot experiment. The isolated strains MS4 and TS1 were found to be most effective and competitive for alfalfa and clover respectively. Overall recovery of the inoculated strains was 30% for alfalfa and 100% for clover as determined by fluorescent antibody technique. MS4 and TS1 are potent strains for the production of biofertilizer for fodder legumes.

Introduction

Rhizobium legume symbiosis is recognized as one of the most important nitrogen fixing system. The isolation of superior *Rhizobium* is very important because the effective rhizobial strains are used as inoculants to ensure effective nodulation (Moxley *et al.*, 1986; Asad *et al.*, 1991; Shah *et al.*, 1995). The competitive ability of an inoculant strain is a major factor determining the success of rhizobial inoculation.

Inoculated *Rhizobium* sp. often fails to compete with indigenous soil rhizobia and do not increase nodulation as these strains have to compete with native rhizosphere community for nutrients (Bromfield *et al.*, 1986; Singleton & Tavares 1986). Therefore, effective inoculant strains have been selected which are able to compete with the native rhizobia and thus form a high percentage of nodulation (Hafeez *et al.*, 1991; Hafeez *et al.*, 2000; Hafeez *et al.*, 2001). *Medicago sativa* (alfalfa) and *Trifolium subterraneum* (clover) are most important forage legumes in Pakistan. Successful establishment of alfalfa and clover involve effective symbiotic association between *Rhizobium* strains and compatible host plants. Inoculation with superior *Rhizobium* strains is required to increase the yield of legume crops through nitrogen fixation (Ather 1988) and also to cut the input price of these fodder crops. Host plant competing rhizobial strains show their high specificity for the respective crops (Hafeez *et al.*, 2000).

The present study is therefore, designed to isolate the most specific, effective and competitive strains for *Medicago* sp., and *Trifolium* sp., and to use them as inoculants.

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Materials and Methods

Collection of plants and *Rhizobium* strains: *Medicago sativa* and *Trifolium subterraneum* plants were collected from the adjacent fields of NIBGE, Faisalabad. The exotic *Rhizobium meliloti* strain TAL-1373 and *Rhizobium leguminosarum* bv. *trifolii* strain TAL-1827 & E-11 were obtained from the BIRCEN, Culture Collection NIBGE, Faisalabad, Pakistan. The seeds of alfalfa and clover plants were obtained from the Ayub Agriculture Research Institute (AARI), Faisalabad, Pakistan.

Isolation and effectiveness of indigenous *Rhizobium* strains: Roots of alfalfa and clover plants were washed and nodules were separated. Nodules were surface sterilized with 0.1% $HgCl_2$. The crushed nodule extract was streaked on yeast mannitol (YEM) agar plates having congo red as indicator (Shah *et al.*, 1995). After the growth appeared on the plates, the cultures were purified by sub-culturing on separate plates by picking single colony on the basis of morphological differences. The pure cultures were confirmed by Gram's staining technique (Rao, 1999) and then grown in YEM broth medium having bromothymol blue (BTB), (Vincent, 1970). The purified cultures were authenticated by their infectivity to their host plants grown in vermiculite and agar glass tubes containing N-free Hoagland nutrient solution (Arnon & Hoagland 1940). Treatments, inoculated and uninoculated were used with three replicates each (Table 2). The glass tubes were kept in growth room at 30°C. The inoculum was given as 1mL.of broth culture per tube (10^9 cells ml^{-1}). Four weeks old nodules were picked and incubated with acetylene for 1 hour at room temperature to determine the nitrogenase activity. (Hafeez *et al.*, 1995). Two controls were used for the assay: 1) without any nodules, containing pure acetylene only; 2) root nodules with acetylene. Trace Gas Chromatograph–GC 2000 (Thermo Quest–C.E instrument Italiana) with a hydrogen flame ionization detector (FID) was used for acetylene reduction assay. The unchanged acetylene and the ethylene produced were calculated as ratio on chrome card software. The nitrogenase activity was expressed as nmoles of C_2H_4 produced h^{-1} g^{-1} nodule dry weight.

C-source utilization: The rhizobial strains isolated from plants were tested for their capability of utilizing different carbon sources. In yeast-mannitol medium (Vincent, 1970) arbinose, xylose, glucose, glactose, raffinose, sucrose, maltose, mannitol and molasses were used as alternative carbon source for mannitol. The concentration of the carbon source was 1% (w/v). Each chemical was filter sterilized by passing through membrane filters 0.2μm size (Millipore Corp). The isolates were streaked on plates in triplicate and the presence or absence of their growth was observed after 3-5 days.

Indigenous population: The population density of indigenous *Rhizobium* sp., from two soils (Table 1) was determined by plant infection technique (Vincent, 1970) using growth pouches containing N-free nutrient solution. The soils were selected on the basis of their cropping history. In soil 1 the fodder legumes had been grown for the past few years whereas no legume had been grown in soil 2. Some of the soil properties are given in Table 1. The seeds were surface sterilized with 0.1% $HgCl_2$ and sterile water and grown in growth room at 30 ± 2°C. Serial four fold dilutions were prepared from both soils and 1 ml of each dilution was applied to the roots of the test plants with four replicates for each dilution. Nodule observations were made daily and data were collected after 30 days of planting. Positive results were compared with a standard most probable number table (Vincent, 1970).

Table 1. Some physiochemical properties of soils and the estimation of population of indigenous *Rhizobium* sp., in two soils.

Soil Type	pH	CEC (c mol kg^{-1})	Organic C (%)	Total N (%)	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	Mineralizable N (mg kg^{-1})	Estimated cells of *Rhizobium* sp. g^{-1}soil	
								M. sativa	*T.subterraneum*
Soil 1 (NIAB) Sandy Loam	7.2	9.0	0.65	0.07	18.0	2.3	1.5	1.1	13.0
Soil 2 Loam (NIBGE)	7.5	10.2	0.50	0.06	14.5	1.2	1.0	13.0	1.5

CEC = Cation exchange capacity.

Annual rain fall = 310.9 ± 47.5 mm

Table 2. Effect of rhizobial inoculation on growth, nodulation and N_2-fixation on *Medicago sativa* and *Trifolium subterraneum*.

Strains	Nodule number plant^{-1}	nodule dry weight plant^{-1} (mg)	ARA nmole of C_2H_4 produced h^{-1} g^{-1} nodule dry wt.
Medicago sativa			
MS1	8 a	3.53 a	266 e
MS2	4 bc	1.17 d	646 b
MS3	5 b	1.33 cd	424 c
MS4	3 c	1.4 c	673 a
TAL-1373	8 a	3.13 b	300 d
Uninoculated (control)	-	-	-
Trifolium subterraneum			
TS1	3 c	2.33 d	303 a
TS2	7 a	3.67 b	231 c
TS3	5 b	3.1 c	273 e
TS4	8 a	4.27 a	243 d
TS5	7 a	3.6 b	218 f
TAL-1827	4 bc	3.2 bc	279 b
E-11	3 c	2.07 d	228 c
Uninoculated (control)	-	-	-

The values given in table are averages of 3 replications and were compared with Duncan's MR test at P=0.05

Competition experiment: The competitive experiment was conducted between the indigenous and inoculated strains. The strains selected for alfalfa plants were MS4 (local) and TAL-1373 (exotic) and for clover plants TS1 (local) and TAL-1827 (exotic). The experiment was conducted in pots containing 4 kg soil. Soil was collected from two locations by mining the soil up to 20 cm, after removing litter and top 1 cm of soil; the soil was air dried and sieved (0.5 cm). The experiment was performed in a completely randomized block design with four replicates and three treatments i.e. inoculated, uninoculated and nitrogen (N) control. N was applied at 60 mg N g^{-1} soil. The seeds were inoculated with a carrier based inoculum. The number of viable rhizobial cells was 10^4 per seed at the time of sowing. Each pot contained 10 seeds. The experiment was conducted in growth room at 30 ± 2°C. The surface of pot was covered with gravel to prevent cross contamination between treatments. The plants were harvested 8 weeks after plantation and nodulation data were recorded. The dry weight and total N was determined in shoots (Bremner, 1965). Nodule occupancy was studied by the method of Schmidt *et al.*, (1968) with the respective fluorescent antibodies of the inoculated strains. Fluorescent antibodies were developed against the inoculated strains, MS4 and TS1 in six months old albino female rabbits while FA's for exotic strains TAL-1373 and TAL-1827 were obtained from NifTAL Hawaii USA.

Results and Discussion

All the strains showed growth in 3 days and turned the yeast mannitol agar media containing bromothymol blue to yellow color showing that all were fast growers and acid producer (Shah *et al.*, 1995). The rhizobial strains did not absorb the color of congo red. The colonies were gummy, circular, convex with smooth edges, glistening translucent or white. All the isolates were Gram negative and rod shaped (Shah *et al.*, 1995; Vincent 1970; Keyser, 1982; Anand & Dogra, 1991).

Table 3. Comparison of exotic and indigenous rhizobial strains in nodulation, growth and N- concentration in *Medicago sativa* and *Trifolium subterraneum*.

M. sativa

	Soil 1				Soil 2			
	INO		UINO	NC	INO		UINO	NC
	MS4	TAL1373			MS4	TAL1373		
Nodule number (plant^{-1})	8.5a	7.2a	7.1a	-	14.5a	12.2a	8.5b	-
Dry weight nodule (mg plant^{-1})	1.3a	1.0a	1.5a	-	7.0a	5.3a	3.2b	-
Dry matter (g plant^{-1})	0.30a	0.35a	0.40a	0.45a	0.50a	0.52a	0.49a	0.58a
Total N (mg plant^{-1})	15.0a	12.5b	14.0b	16.5a	12.5a	10.5b	7.9b	13.5a

T. subteranneum

	Soil 1				Soil 2			
	INO		UINO	NC	INO		UINO	NC
	TS1	TAL1827			TS1	TAL1827		
Nodule number (plant^{-1})	26.5a	22.5a	8.5b	-	18.5a	16.2a	7.5b	-
Dry weight nodule (mg plant^{-1})	10.2a	8.1a	2.2b	-	7.8a	5.8a	2.5b	-
Dry matter (g plant^{-1})	0.50a	0.53a	0.48a	0.57a	0.33a	0.28a	0.35a	0.32a
Total N (mg plant^{-1})	17.0a	14.5b	13.0b	18.5a	16.1a	15.1a	13.7b	15.5a

Mean of four replicates. Numbers between rows followed by the same letter are not significantly different at P=0.01 by Duncan's Multiple Range Test; INO, Inoculated; UINO, Uninoculated; NC, Control, *M, Medicago*; T., *Trifolium*

Table 4. Relative abilities of inoculated strains to nodulate the fodder legumes in two soils.

	Strain number	Nodule occupancy (%age)	
		Soil 1	Soil 2
Medicago Sativa	MS4	78	100
	TAL1373	67	60
Trifolium subterraneum	TS1	82	73
	TAL 1827	30	50

Utilization of different carbon sources is an effective tool to characterize the isolates (Hafeez *et al.*, 1993; Moawad & Bahlool, 1993; Monza *et al.*, 1992; Roderguez *et al.*, 1987). The results showed that all the isolates utilized a wide range of carbon sources. Full growth was observed in rhizobial strains MS1, TAL-1373, TS3 and TS4 with respect to all C- sources. This confirmed that fast growers can oxidize a wide range of carbon sources as sole carbon source (Hafeez *et al.*, 1993).

Five out of eight strains isolated from alfalfa plants were highly effective. The data showed that the number of nodules formed by local strains MS1, MS2, MS3 and MS4 were 8, 4, 5 and 3 respectively, while exotic strains TAL 1373 formed 8 nodules per plant. Variations were also observed in dry weight of nodules formed by different strains. The strain MS1 showed maximum dry weight 3.5 mg plant^{-1} as compared to that of TAL-1373 i.e. 3.1 mg plant^{-1}. The *R. meliloti* strains showed variations in their effectiveness on alfalfa plants (Table 2).

The maximum nitrogenase activity was 673 n moles of C_2H_4 produced $h^{-1}g^{-1}$ nodule dry weight of nodules showed by MS4 whereas, the minimum nitrogenase activity was 266 n moles of C_2H_4 produced $h^{-1}g^{-1}$ nodule dry weight showed by MS1 (Table 2).
Seven out of eleven strains isolated from the clover plants were highly infective and effective. The number of nodules formed by locally isolated strains TS1, TS2, TS3, TS4 and TS5 were 3,7,5,8 and 7 respectively, while exotic strains TAL-1827 and E11 formed 4 and 3 nodules per plant respectively. The maximum dry weight was observed in the local strain TS4 (4.3 mg. per plants) as compared to exotic strain E-11which showed minimum (2.1mg. plant^{-1}) dry weight of nodules (Table 2). The specific nitrogenase activity ranged from 218-303 n moles of C_2H_4 produced $h^{-1}g^{-1}$ dry weight of nodules.

Competition experiment: The response to inoculation varied among legume species and between soils (Table 3). The nodulation response was more frequent in the soil 2 where no previous leguminous crop had been grown indicating that this soil was more receptive to introduced inoculants. *T. subterraneum* respond significantly to the inoculation, with increased nodulation in both soils, similarly significant response was obtained with *M. sativa* even though the population of indigenous strain was not so high. These results are similar to the findings of other authors (Singleton & Tavers, 1986; Asad *et al.*, 1991; Thies *et al.*, 1991; Hafeez *et al.*, 2001), that relatively small indigenous population of rhizobia is sufficient to meet the host N demand.

Strain identification in nodules from the inoculated treatments indicated the competition between the inoculated and indigenous strains (Table 4). Nodule occupancy by inoculant strains ranged from 30 to 100%. The local strains MS4 isolated from *M. sativa* and TS1 isolated from *T. subterraneum* found to be highly effective and competent than the exotic strains TAL-1373 and TAL-1827. It could be inferred from these results

that all the isolates from single locality were not equally efficient. Thus the selection of highly effective and competitive strain as inoculum is very important to increase the amount of fixed nitrogen and hence the yield of the crop. It has been reported that certain species of *Trifolium* and *Medicago* differ in their selection of rhizobia (Russel & Jones1975; Harderson *et al.*, 1982; Bromfield 1984; McLoughlin & Durican 1985) and inoculation has significant effects on nodulation, dry weight of nodules and nitrogenase activity. It was found that the local strains of both the alfalfa and clover were more effective than the exotic strains. There was a positive correlation (r = 0.87) between nodule number and nodule dry weight but poor correlation was found between nodule number and ARA (r=0.33) and nodule dry weight and ARA (r= 0.62) in rhizobial strains isolated from alfalfa plants. Similarly strains of clover showed positive correlation (r= 0.84) between nodule number and nodule dry weight. The correlation between nodule dry weight and ARA was 0.82 and between nodule number and ARA was 0.68. These results indicate that nitrogen fixing ability of a strain depends on the effectiveness of the strains and not on the number of nodules (Shah *et al.*, 1995; Hafeez *et al.*, 2000). On the basis of above mentioned results the local strains MS4 and TS1 are being used for the production of bio-fertilizers (*Bio Power*) for alfalfa and clover crops and is being supplied to the local farmers to be used in the field.

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