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Potential for inoculation of common bean by effective rhizobia in Tunisian soils

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Summary – *Phaseolus vulgaris* (L) is poorly nodulated in all the regions of Tunisia where this crop is grown. Selection of effective strains needs to be developed. An effectiveness test trial was carried out on Royalnel and Coco cultivars in hydroponic aseptic conditions for seven reference strains and three local isolates of common bean rhizobia. A significant interaction strain × cultivar was observed and, unlike local isolates, most reference strains were very effective. The ELISA technique using polyclonal antibody was used to evaluate nodule occupancy of inoculated common bean rhizobia in Tunisian soils. Rabbit antisera were raised against one local isolate (HB) and five reference strains (CIAT899, USDA2667, USDA2669, USDA2676 and IRATYH112). Analysis of relatedness between 44 strains and isolates showed that 77% of the local isolates did not react with any of the sera used, whereas the remaining isolates were distributed into three serogroups. An inoculation experiment with the selected reference strains CIAT899, USDA2667 and IRATYH112, which are serologically distinguishable from native rhizobia, was performed in a green house for Royalnel and Coco cultivars grown in soil cores from two sites (Boucharraï and Chrifet). Nodule occupancy of the inoculated strains was assessed and concomitant effect of inoculation on nodule number and shoot nitrogen content was analysed. A highly significant treatment × cultivar × site interaction was observed for the parameters studied. While in Boucharraï soil inoculation leads to a significant improvement of shoot nitrogen content for Royalnel cultivar, inoculation of the same cultivar in Chrifet soil leads to an increase in nodulation by native rhizobia without improvement of plant growth. For the two cultivars, N-fertilization leads for the two cultivars to a decrease in nodule number, particularly in Chrifet soil. Thus, to improve establishment of symbiotic process, the use of N-fertilizer should be rationalized.

inoculation / rhizobia / *Phaseolus vulgaris* / ELISA

Résumé – Faisabilité d'inoculation du haricot par des *Rhizobium* efficients en sols tunisiens. Le haricot (*Phaseolus vulgaris* (L)) est faiblement nodulé dans toutes les régions de la Tunisie où il est cultivé. Pour sélectionner des souches efficientes un essai a été mené en système aseptique de culture hydroponique. Une interaction significative souche × cultivar a été observée. Contrairement aux isolats locaux la plupart des souches de référence sont très efficientes. La méthode Elisa avec des anticorps polyclonaux a été utilisée pour évaluer l'occupation nodulaire des *Rhizobium* spécifiques du haricot inoculés dans les sols tunisiens. Des antisérums de lapin ont été préparés contre un isolat local (HB) et cinq souches de référence (CIAT899, USDA2667, USDA2669, USDA2676, and IRATYH112). L'analyse de l'apparentement entre 44 souches et isolats montre que 77 % des isolats locaux ne réagissent pas avec ces sérums alors que les bactéries restantes forment trois sérogroupes. Une expérience d'inoculation avec les souches de référence sélectionnées CIAT899, USDA2667 et IRATYH112 distinguables sérologiquement des *Rhizobium* natifs, a été conduite en serre sur les cultivars Royalnel et Coco cultivés sur des carottes de sols provenant de deux sites différents (Boucharaï

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et Chrifet). Le pourcentage de nodosités formés par les souches inoculées a été estimé et l'effet de l'inoculation sur le nombre de nodules et l'azote dans les parties aériennes ont été analysés. Une interaction hautement significative traitement × cultivar × site a été observée pour les paramètres étudiés. Alors que sur le sol de Boucharraï l'inoculation conduit à une amélioration significative du contenu en azote des parties aériennes pour le cultivar Royalnel, l'inoculation du même cultivar sur sol de Chrifet conduit plutôt à une stimulation de la nodulation par les bactéries natives sans amélioration de la croissance végétale. L'application d'engrais azoté conduit pour les deux cultivars à une diminution de la nodulation particulièrement sur sol de Chrifet. En vue de favoriser l'établissement du processus symbiotique, l'utilisation de ce composé doit être rationalisée.

inoculation / *Rhizobium* / *Phaseolus vulgaris* / Elisa

INTRODUCTION

Nitrogen fixation by legume-*Rhizobium* symbiosis is important to agricultural productivity and is therefore of great economic interest. Like many other food legumes, *Phaseolus vulgaris* (L), is generally grown in rotation with cereals. However, this food legume is known to exhibit a low ability to fix nitrogen (Larue and Patterson, 1981; Graham, 1981, 1992). This is perhaps partly due to the absence of appropriate rhizobial strains and to environmental variables (Smith et al, 1987; Bottomley, 1992). Indeed, for a long time, common bean varieties have been selected for soils with a high N-content in developed countries. Defect in nitrogen fixation by the common bean will also affect soil nitrogen balance and growth of the subsequent crop (Keatinge et al, 1988). Lack of efficient nodulation in Tunisian soils has been observed for a long time (Dahmane et al, 1995), and this has perhaps favoured the wide use of N-fertilizer for this crop.

A technique commonly used to improve legume growth, pod and grain yield, is inoculation with *Rhizobium* spp strains selected on the basis of their effectiveness. Nevertheless, these strains often fail to nodulate because they are unable to withstand environmental stress factors and/or to compete with well-adapted native rhizobia strains (Thies et al, 1992). Therefore, to identify rhizobia occupying nodules, and assess competitiveness, a reliable and sensitive method of identifying specific strains in nodules is required. This can be accomplished by a number of procedures. Serotyping is the most commonly used method for *Rhizobium* identification (Kishinevsky and Bar-Joseph, 1978; Ayanaba et al, 1986; Cleyet-Marel, 1987; Wolff et al, 1991).

In the present work we used ELISA (enzyme-linked immunosorbent assay) for studying the relatedness between indigenous common bean rhizobia isolated from Tunisian soils and six reference strains. The usefulness of this technique in discriminating between reference and native rhizobia was evaluated. The symbiotic effectiveness of the six reference strains was measured on two common bean cultivars under laboratory conditions. Soil cores from two sites in the Cap Bon region (Chrifet and Boucharraï) were inoculated with these strains and nodule occupancy of the inoculant strains was assessed.

MATERIALS AND METHODS

Biological material

The origin and source of common bean *Rhizobium* are described in table I. Reference strains and local isolates were grown and maintained on YMA (yeast-mannitol-agar) medium (Vincent, 1970). Two cultivars of *Phaseolus vulgaris* (L) were used in this work. They are grown by local farmers, Royalnel for green beans and Coco cultivar for grain production.

Effectiveness test

Ten rhizobia strains and isolates (table I) were tested in a preliminary experiment for symbiotic effectiveness on Coco and Royalnel cultivars. This experiment was performed using an aseptic N-free hydroponic gravel culture system (Beck et al, 1993) in the glass house (using natural daylight, during March and April, with a temperature of 28 °C by day and 15 °C by night). The 20 treatments (ten strains × two cultivars) were replicated eight times in sterile disposable 0.5 L plastic cups (one plant/cup) three quart full of sterile gravel. Surface sterilized seeds were pregerminated in agar 0.9%, sown in pots, and irrigated with the modified sterile N-free nutrient solution of Broughton and Dillworth (Somasegaran and Hoben, 1985) (Fe(II)EDTA instead of Fe-citrate). The nutrient solution was provided to

Table I. Sources of common bean rhizobia.

Reference strains and local isolates	*Origin*	*Source*
Reference strains:		
GB9.6*	Gembloux, Belgium	FSAG, C Bonnier
CIAT899*	Colombia	CIAT
IRATYH112*	Rwanda	CIRAD, P Beunard
USDA2667*	Washington	USDA, P Van Berkum
USDA2668*	–	USDA, P Van Berkum
USDA2669*	–	USDA, P Van Berkum
USDA2676*	Colombia	USDA, P Van Berkum
Local isolates:		
HB*,15c3,16c1, 18b1, 20c1,	North of Tunisia	This study
23c1, 25c2, 28b3, 28c2, 30c2,	North of Tunisia	This study
31a2, 31c3, 33a3, 33c3, 34b2,	North of Tunisia	This study
34c2, 35a1, 35b2, 35c1, 35c2,	North of Tunisia	This study
36b3, 36c1, 37b1, 37c2, 39b2	North of Tunisia	This study
107*, 108*,1b3, 2b1, 2b3,	Cap Bon, Tunisia	This study
3b1, 3b3, 6b1, 6b3,7a3, 7c1,	Cap Bon, Tunisia	This study
8a3, 9b1, 10a3, 12a3, 12b2,	Cap Bon, Tunisia	This study

* Strains and isolates used in the effectiveness test trial. FSAG: Faculté des sciences agronomiques de Gembloux, Belgium. USDA: United States Department of Agriculture, Beltsville, MD, USA. CIAT: Centro Internacional de Agriculture Tropicale, Cali, Colombia. Cirad: Centre de coopération internationale en recherche agronomique pour le développement, Montpellier, France.

each cup by an automatic syringe. Plants were inoculated with 1 mL of rhizobial culture containing 10^9 bact/mL at seedling emergency and irrigated alternately with a N-free solution or sterile distilled water. In addition, two non-inoculated controls, one receiving 100 ppm NO_3-N in the nutrient solution (N-control), the other only the N-free solution (0N-control), were added to the trial. This last control permitted us to verify the sterility of the culture system and to evaluate the residual growth using only seed N. Six weeks after sowing, plants were harvested. Shoots were excised; then, nodules were enumerated and detached from their roots. All these parts were oven-dried, weighed and total nitrogen was determined (Bremner, 1965).

Preparation of antigens and antisera

Five reference strains (CIAT899, USDA2667, USDA2669, USDA2676 and IRATYH112) and one local isolate (HB) were grown on YMA for 3 days. Culture was then harvested in phosphate buffer saline (PBS) and vortexed. Numbers of bacteria were estimated by using plate counting procedures (Vincent, 1970). Cells were centrifuged and washed three times and resuspended in sterile NaCl (0.85%) solution and diluted to 10^9 bact/mL to immunize rabbits with similar amounts of antigens. Suspensions were dispensed in sterile bottles and stored at 4 °C until immunization. In accordance with the schedule described by Beck et al (1993), antisera were obtained from young adult New Zealand white rabbits. After each bleeding, serum blood fraction was isolated and stored at –20 °C.

Relatedness between common bean rhizobia

Serological relatedness of common bean *Rhizobium* strains and isolates was assessed using the six sera described above. Fresh *Rhizobium* culture on YMA slant was suspended in PBS and adjusted to the same turbidity (A_{620} = 0.20) and diluted 50-fold before use with the coating solution (5 mM carbonate buffer to pH 9.6). Microtiter plates, 96 wells (Immulon II) were used. Indirect ELISA was performed as described by Beck et al (1993) with an automatic ELISA plate reader (Multiscan MCC/340) according to the following.

i) Tested bacteria were added to the wells (100 µL/well), and incubated for 90 min at 37 °C.

ii) Plates were then washed ten times with PBS containing 0.1% Tween 20 (100 µL/well).

iii) In order to ensure complete inhibition of non-specific binding, PBS Tween 20 containing 0.5% bovine serum albumine was added to the wells (200 µL/well) and incubated for 90 min at 37 °C.

iv) Step ii) was repeated.

v) An amount of 100 µL of antisera diluted in PBS was added to the wells. The titers used were 1/500 for USDA2667, 1/5 000 for CIAT899 and HB, and 1/10 000 for USDA2669, USDA2676 and IRATYH112. Plates were incubated for 90 min at 37 °C and for 30 min at 4 °C.

vi) Step ii) was repeated.

vii) Goat anti rabbit IgG conjugated with peroxydase (Biorad) was diluted 1 000-fold in PBS and added (100 µL) to the wells.

viii) Step ii) was repeated.

ix) OPD (o-phenylenediamine, Biorad) was used as a substrate (0.4 mg/mL in 0.1 M citric acid phosphate buffer, pH 5.5). Immediately before use, 8 µL of fresh 35% hydrogen peroxide were added per 20 mL of substrate. In this study, 200 µL of this solution were added to each well.

x) The enzyme reaction was stopped after 15 min by adding 50 µL of 2 M H_2SO_4 to each well.

The optical densities were measured at 492 nm. On each plate the optical density of the substrate was measured in wells where no antigen or serum was used as controls and values below 0.05 were normally found. For the reactivity between strains or isolates and antisera, ELISA absorbance values (492 nm) were normalised against the appropriate positive control as described by Eaglesham and Sinclair (1988). Results from different experiments could thus be compiled into a single data set.

To ensure accuracy of the positive control data, five replicate determinations were made. For other tests, three replicates were performed.

Intact soil core experiment

Two sites harbouring common bean specific rhizobia were selected to analyse the inoculation effect of three reference strains (CIAT899, USDA2667 and IRATYH112) on two cultivars. Soil samples were collected from these two sites in heavy-duty PVC plastic pipes (diameter = 15 cm, height = 25 cm). The main characteristics of soils are given in table II. Bean seeds were sown on these soil cores, watered and protected from contamination as indicated by Beck et al (1993). When they emerged, seedlings were thinned to three and each plant was inoculated with 1 mL of rhizobial culture containing 10^9 bact/mL. Five cores were sown per treatment. Irrigation was carried out using sterile water. For the N-control treatment the equivalent of 120 kg N/ha (in urea form) was added: half 1 week after seedling emergence and half 5 weeks after planting. Plants were harvested at the mid-anthesis stage after 8 weeks. Shoots were excised, oven-dried and weighed and the total nitrogen analysed (Bremner, 1965). Nodules were enumerated and samples from various treatments were conserved in tubes with anhydrous $CaCl_2$ and stored at 4 °C until ELISA analysis was performed.

The nodule occupancy study was performed on samples of eight nodules randomly selected from inoculated plants of each soil core. Thus, 40 nodules were analysed by treatment. Nodule occupancy was determined by indirect ELISA according to the methodology of Beck et al (1993). The absorbance was read at 492 nm and compared to the blanks and positive controls.

Data analysis

The individual and combined analyses of variance over the two cultivars were performed on the data of the effectiveness trial and the soil core experiment using SAS Software (1994). Then, least significant differences (LSD) were calculated to determine statistical differences between treatments.

RESULTS

Evaluation of strain effectiveness

This study was performed to identify efficient strains and isolates. Thus, analysis of variance on data obtained from effectiveness test trial was carried out for the treatment and cultivar factors and their interaction (table III). This analysis showed that the differences between treatments were statistically significant for all studied characteristics. Between cultivars, there was one difference for nodule number and nodule dry weight. Royalnel

Table II. Some chemicals and physical properties of the soils used.

Soils	*Boucharrai*	*Chrifet*
pH H_2O	8.23	8.13
P_2O_5 ppm[a]	18.9	19.3
Total soil N (%)[b]	0.042	0.105
Clay (%)	14	14
Silt (%)	4	2
Sand (%)	82	84
K_2O ppm	145	88
Organic matter (%)	0.81	0.99

[a]Olsen and Dean (1965); [b]Bremner (1965).

Table III. Analysis of variance for shoot dry weight (SDW), nodule number (NN), nodule dry weight (NDW), shoot nitrogen (SN) and total nitrogen (TN) in Coco and Royalnel cultivars inoculated with rhizobia (shown in table I) in the effectiveness test trial.

Source of variation	*SDW*	*NN*	*NDW*	*SN*	*TN*
Treatment	6.9[$]***	3.5**	2.6**	3.2***	6.5***
Cultivar	0.3 NS	15.7***	5.6*	3.1 NS	1.8 NS
Cultivar × treatment	1.8*	2.4*	2.0*	2.5**	4.2***

[$]F values, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, NS: not significant.

cultivar showed the highest averages. This cultivar gave 109 nodules/pl and 75 mg nodule dry weight/pl. Averages for Coco cultivar were, respectively, 75 and 64 for the same parameters. For other characters, the difference between cultivars was probably hidden by treatment × cultivar interaction. In fact this interaction was significant for all the characteristics (table III). Then, the analysis of results was performed separately for the two cultivars (table IV). It appeared that for example strain USDA2669 showed SDW (shoot dry weight), SN (total shoot nitrogen) and TN (total plant nitrogen) on Coco cultivar means higher than those observed on Royalnel cultivar. On the other hand, USDA2676 appeared to be more effective on Royalnel cultivar. According to the results obtained with the strain × cultivar associations (table IV) and when compared with non-inoculated 0N- and N-control, the studied strains can be grouped according to their effectiveness as shown by SDW, SN and TN on the two cultivars; group i) highly efficient strains: CIAT899, USDA2667, USDA2669, USDA2676 and IRATYH112; group ii) slightly efficient strains: GB9.6, USDA2668; and group iii) inefficient isolates: HB, 107 and 108.

Correlation analysis (data not shown) indicated that SDW, SN and TN, followed similar patterns and showed significant positive correlation. These parameters could be considered as accurate criteria for symbiotic activity analysis on common bean and then could be used for screening of further strain × cultivar treatments for their symbiotic effectiveness in sterile cup system.

Serological relatedness of the common bean rhizobia strains and isolates

Reaction of pure culture from 44 reference strains and local isolates was analysed with the six antisera. The results revealed a wide serological relatedness between the four reference strains CIAT899, USDA2669, USDA2676 and IRATYH112 (table V). These strains form one group and share therefore several common anti-

Table IV. Mean shoot (SDW) and nodule (NDW) dry weight (mg/pl), nodule number (NN) (nod/pl), shoot (SN) and total (TN) nitrogen (mg/pl) of 6-week-old plants of two common bean cultivars as affected by rhizobial strain treatments under aseptic N-free hydroponic culture.

Cultivar	*Treatment*	*SDW*	*NN*	*NDW*	*SN*	*TN*
Royalnel	0N-control	529			6	9
	HB	581	113	55	6	10
	107	545	73	31	6	10
	108	509	84	35	6	10
	GB9.6	740	136	100	21	30
	CIAT899	755	118	85	23	33
	USDA2667	873	114	88	31	39
	USDA2668	565	138	96	11	18
	USDA2669	702	102	77	22	32
	USDA2676	1021	105	94	32	43
	IRATYH112	775	109	86	24	33
	N-control	978			31	43
	LSD (0.05)	132	16	11	2	5
Coco	0N-control	604			8	15
	HB	538	43	28	8	13
	107	593	48	35	7	14
	108	596	35	21	9	15
	GB9.6	588	87	81	19	28
	CIAT899	754	97	75	25	37
	USDA2667	743	78	66	30	40
	USDA2668	602	81	90	13	21
	USDA2669	707	109	84	25	35
	USDA2676	595	83	79	21	31
	IRATYH112	699	85	83	23	33
	N-control	859			29	43
	LSD (0.05)	110	13	8	2	4

Table V. ELISA cross-reaction of six antisera to their related strains and 38 local common bean rhizobia isolates.

	Antisera					
Bacteria	*USDA 2667*	*CIAT 899*	*USDA 2669*	*USDA 2676*	*IRATYH 112*	*HB*
Reference strains:						
USDA2667	1.00*	–§	–	–	–	–
CIAT899	–	1.00	0.98	0.93	0.89	–
USDA2669	–	0.90	1.00	0.87	0.78	–
USDA2676	–	1.21	1.18	1.00	0.98	–
IRATYH112	–	0.97	0.97	0.97	1.00	–
Local isolates:						
HB	–	–	–	–	–	1.00
31c3	–	–	–	–	–	0.97
30c2	–	–	–	–	–	0.95
33a3	–	–	–	–	–	0.83
36c1	–	–	–	–	–	0.55
18b1	0.33	–	–	–	–	–
16c1	0.72	–	–	–	–	–
34c2	0.76	–	–	–	–	–
35c2	0.66	–	–	–	–	–
35c1	0.29	–	–	–	–	–
29 other local isolates†	–	–	–	–	–	–

* Normalized absorbance values (NAV). § indicates less than 0.1 NAV. † 15c3, 20c1, 23c1, 25c2, 28b3, 28c2, 31a2, 33c3, 34b2, 35a1, 35b2, 36b3, 37b1, 37c2, 39b2, 1b3, 2b1, 2b3,3b1, 3b3, 6b1, 6b3, 7a3, 7c1, 8a3, 9b1, 10a3, 12a3, 12b2.

genic components even if they come from distant geographic regions. However, wide serological differences have been observed between these strains, the local isolates or reference strain USDA2667. In addition, as shown in table V, strain USDA2667 forms its own group and cross-reacts with five local strains (18b1, 16c1, 34c2, 35c2, 35c1) from the northern region of Tunisia with normalised absorbance values (NAV) ranging from 0.29 to 0.76. The local strain (HB) for which an antiserum was produced, cross-reacts with four local isolates (table V) originated from the north region (31c3, 30c2, 33a3, 36c1) with NAV ranging from 0.55 to 0.97.

Nodule occupancy of introduced strains and their effect on nodulation and plant growth

The usefulness of indirect ELISA for the nodule occupancy study of three efficient reference strains (CIAT899, USDA2667 and IRATYH112) on Royalnel and Coco cultivars in Tunisian soils was shown by the analysis of nodules formed by native rhizobia. The analyses of samples of 30 nodules from the two cultivars grown on the soils of Boucharrai and Chrifet showed that none of the antisera raised against the three strains reacted significantly with nodule extracts from nodules formed by native rhizobia. Thus, it was possible to distinguish easily between nodules containing the inoculated strain and those occupied by native rhizobia.

Thereafter, the performance of these strains as inoculants was analysed in a soil core experiment. The effect of inoculated reference strains on soil core grown common bean was studied on SN (shoot nitrogen), NN (nodule number) and %NO (percentage of nodule occupancy). Analysis of variance for data of this experiment was performed for the treatment, cultivar and site factors and their interactions. For all parameters, except for SN with site factor (table VI), the results showed highly significant differences between treatments, cultivars and sites. Highly significant interactions between treatment, cultivar and site factors were also obtained for all parameters except for SN content with cultivar × treatment interaction.

Comparative analysis of treatments in every site and for the two cultivars is shown in table VII.

Table VI. Analysis of variance for shoot nitrogen (SN), nodule number (NN) and percent nodule occupancy (%NO) in Coco and Royalnel cultivar grown on soil cores of Boucharrai and Chrifet with (N-control) or without (0N-control) N-fertilizer or inoculated with reference strains (CIAT899, USDA2667 and IRATYH112).

Source of variation	*SN*	*NN*	*%NO*
Site	0.5[$] NS	104.4***	304.0***
Cultivar	14.5***	438.2***	94.5***
Treatment	6.5***	196.4***	12.9***
Site × cultivar	10.3**	64.5**	52.6***
Cultivar × treatment	1.9 NS	11.2***	25.6***
Site × treatment	3.1**	7.6***	3.6***
Site × cultivar × treatment	3.4**	4.9***	43.2***

[$]F values, ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$, NS: not significant.

For shoot nitrogen content, inoculated treatments of Royalnel cultivar grown on soil cores from Boucharraï site gave significantly higher means than that of 0N-control (table VII). In the same site, Coco cultivar seemed to be less affected than Royalnel cultivar by nitrogen deficiency. In fact, no significant difference was observed between all treatments for that cultivar (table VII). From the results of Chrifet soil cores, nitrogen appeared not to be a limiting factor in this soil for Royalnel cultivar. Consequently, inoculation did not lead to a noticeable improvement of shoot nitrogen. Moreover, Royalnel cultivar inoculated with USDA2667 and IRATYH112 showed significantly lower averages for shoot nitrogen than that of 0N-control (table VII). In the same site for Coco cultivar, inoculation did not lead to any improvement of SN despite the application of N-fertilizer.

Nodulation, as estimated by NN, was generally improved by inoculation with reference strains. Except for Coco in Chrifet soil inoculation has led to a significant improvement of at least 48% for CIAT899 in Chrifet for Royalnel cultivar, and reached 73% for IRATYH112 in Boucharraï soil for Coco cultivar (table VII). On the contrary, the use of urea as N-fertilizer reduced nodule number to about 30% in Boucharraï soil and to more than

Table VII. Mid anthesis sampling data from inoculated soil core experiment.

Site	*Cultivar*	*Treatment*	*SN*	*NN*	*%NO*
Boucharraï	Royalnel	N-control	79	47	–
		CIAT899	67	116	93
		USDA2667	61	120	65
		IRATYH112	62	116	83
		0N-control	34	80	–
		LSD (0.05)	11	12	14
	Coco	N-control	62	42	–
		CIAT899	64	100	43
		USDA2667	58	98	23
		IRATYH112	57	102	38
		0N-control	60	59	–
		LSD (0.05)	NS	13	10
Chrifet	Royalnel	N-control	77	30	–
		CIAT899	67	109	13
		USDA2667	50	121	18
		IRATYH112	60	110	13
		0N-control	71	74	–
		LSD (0.05)	9	8	NS
	Coco	N-control	65	15	–
		CIAT899	9	62	3
		USDA2667	56	61	15
		IRATYH112	49	57	3
		0N-control	52	56	–
		LSD (0.05)	8	9	NS

NS = not significant at 0.05 level. SN: shoot nitrogen (mg/pl). NN: nodule number (nod/pl). %NO: percentage nodule occupancy by inoculated strains.

50% in Chrifet soil (table VII), compared to the 0N-control.

Nodule occupancy by inoculated strains showed that all these strains (table VII) are more frequently present than native rhizobia in nodules of Royalnel cultivar grown in Boucharraï soil. On the contrary, for the two cultivars in the Chrifet site, native rhizobia occupied more nodules than inoculant strains. Nodulation of the two cultivars with inoculant strains seems to be more difficult in Chrifet soil than in the other one.

DISCUSSION AND CONCLUSIONS

Phaseolus vulgaris is an important N-fertilizer-dependent crop in Tunisia and no work was carried out to improve its N_2-fixation capacity in the field. In spite of its well-established need for inoculation (Dahmane et al, 1995), the process of performance strain selection is not yet developed. The first step in this process is the selection of efficient strains either under laboratory, or glass house conditions.

In this work, effectiveness of seven reference strains and three local isolates of common bean rhizobia was assessed for two common bean cultivars (Royalnel and Coco) in a glass house trial. Interaction between strain or isolate and cultivar was significant for all characteristics (table III). This result confirms those obtained by Graham (1981) and Amarger (1986), who showed that nitrogen fixation depends on strain × cultivar interaction and that the process of selection of efficient strains should be developed with adequate cultivars. The choice of the cultivars used should be made according to either shoot dry weight, or nitrogen content of shoot plant. The effectiveness test trial showed that studied local isolates were not efficient (table IV) and confirms observations of Dahmane et al (1995) which indicated that nodules on common bean in field are rare and inefficient. However, Mhamdi (unpublished data) recently identified some local effective isolates on Coco cultivar in an effectiveness test trial.

Antisera production was developed to carry out many ecological studies including competitiveness on rhizobia (Kishinevsky and Bar-Joseph, 1978; Ayanaba et al, 1986; Cleyet-Marel, 1987; Evans et al, 1996). Analysis of the serological relatedness of common bean rhizobia collection, according to six antisera (table V) showed that 77% of local isolates did not react with any of the used antisera, whereas reference strains CIAT899, USDA2669, USDA2676, IRATYH112 and USDA2667 were distinguishable from the majority of local isolates. This fact may suggest that local native rhizobia should contain many particular antigenic components.

Strains CIAT899, USDA2667 and IRATYH112 were selected for their effectivity on the two cultivars in the effectiveness test trial and studied in a soil core experiment. This inoculation trial was carried out in sites where native strains did not cross-react with reference strains.

The results (table VII) suggest that the growth of Royalnel cultivar in Boucharrai soil was limited by nitrogen availability and that it is possible to improve its nitrogen content by inoculation with selected strains. This was not the case for Coco cultivar. These results confirm those of Peterson and Loynachan (1981) and Salez and Saint-Macary (1987) who showed that inoculation can lead to plant N improvement. The very clear preferential nodulation of Royalnel with strains CIAT899, USDA2667, and IRATYH112 to native rhizobia is comparable to what was described on different cultivars of *Glycine* spp (Cregan and Keyser, 1988) and white clover (Mytton, 1975). These reference strains could be good candidates for inoculation of this cultivar in that site for further field experiments. In Chrifet soil, Coco cultivar was also limited by nitrogen availability. Yet, inoculation by reference strains could not improve SN; this may be due to soil conditions including chemical components, higher number of native rhizobia or higher competitiveness of native isolates. As shown by Thies et al (1992), adapted native strains are more competitive than inoculated strains in some environments

Unlike SN, NN was generally increased by inoculation in the trial (VII). This result may be explained by the behaviour of native and introduced populations of rhizobia. Foreign strains in the rhizosphere, irrespective of the formed nodules, seem to favourize infectivity of native adapted specific rhizobia. Indeed, the number of nodules formed by native rhizobia is improved for inoculated Royalnel in Chrifet soil with at least 28%.

Data in table VII confirm the results obtained by many authors (Schuller et al, 1986; Heakman, 1987; Salez and Saint-Macary, 1987; Minchin et al, 1989) who showed that soil nitrogen is inhibitory for the symbiotic process. Surveys for nodulation of field grown bean over Tunisian soils showed rare and ineffective nodules probably because of the use of generally more than 100 kg/ha N-fertilizer. This chemical component

should be taken into account for the improvement of symbiotic nitrogen fixation.

Generally for the different analysed parameters, analysis of inoculation effect trial data (tables VI) showed significant interactions between analysed factors. This fact emphasizes the important role of nitrogen nutrition status which includes plant-associated rhizobia, relation with cultivars and physicochemical components of soil. Then, as shown by Cleyet-Marel and Pinochet (1986), inoculation should not be considered as a panacea. It represents the issue of a long process and one aspect of a total management program.

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