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Chickpea and its root-nodule bacteria: implications of their relationships for legume inoculation and biological nitrogen fixation

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SUMMARY - Chickpeas are able to utilize atmospheric nitrogen derived from the symbiotic relationship formed with root nodule bacteria. This association is highly specific in chickpea, with a unique group of rhizobia necessary for formation of nodules and nitrogen fixation. Nodulation problems attributed to the rhizobial symbiont may be due to absence of appropriate strains, low population numbers, low infectiveness, poor survival in soil, or competition amongst strains of rhizobia. Legume inoculation is a way of assuring that the strain of *Rhizobium* appropriate for the cultivar being planted is present at the proper time in numbers sufficient to assure effective nodulation and nitrogen fixation. Inoculation trials in the region indicate yield response of cultivars to application of appropriate rhizobial strains, but the importance of adequate strain testing prior to an inoculation effort is emphasized.

RESUME - "Le pois chiche et les bactéries qui forment les nodules de la racine: implications de leurs relations sur l'inoculation des légumineuses et la fixation biologique de l'azote". Le pois chiche est capable de s'associer avec des bactéries du sol pour former de nouvelles structures racinaires à l'intérieur desquelles l'azote atmosphérique est réduit en ammonium. Cette association est très spécifique et seul un groupe particulier de Rhizobium peut induire la formation de nodosités et fixatrices d'azote. Les problèmes de nodulation attribués à la bactérie symbiotique peuvent être dus à l'absence de souches spécifiques, à leur faible nombre, à leur pouvoir infectieux, à leur mauvaise survie dans le sol ou à des problèmes de compétition entre souches. L'inoculation des légumineuses est un moyen de s'assurer que la souche de Rhizobium la plus adaptée à un cultivar donné sera présente au bon moment et en nombre suffisant pour promouvoir une nodulation abondante et une bonne fixation d'azote. Des essais d'inoculation indiquent des gains de récolte pour certains cultivars en réponse à l'application de souches appropriées de Rhizobium et l'importance des tests de sélection des souches avant d'envisager l'inoculation à grande échelle est soulignée.

Introduction

Nitrogen is one of the elements essential to the synthesis of amino acids which, in turn, are used by the plant to form protein. Plants primarily take nitrogen in the ionic form of either ammonium (NH^+_4) or nitrate (NO^-_3). Leguminous plants are also able to utilize nitrogen derived from the symbiotic relationship they form with root nodule bacteria. This phenomenon is extremely important and the value of this "free" fertilicer N_2 can be placed in global perspective if one considers that an estimated 50 million tons of nitrogen are manufactured industrially each year against an estimated 90 million tons fixed by plant processes.

Most legumes can provide enough nitrogen for their physiological needs and the use of supplemental nitrogen causes little or no yield increase and may be detrimental to the symbiotic relationship.

Root nodule bacteria are fairly widespread in nature, There are two genera, several species within the genus and many strains have been identified within species (Table 1).

Table 1. Classification of the root nodule bacteria: genera, species and cross-Inoculation groups.

Genus	Species	Biovars	Plant host
Rhizobium	*R.leguminosarum*	*trifolii* *phaseoli* *vicieae*	*Trifolium* *Phaseolus* *Pisum, Lathyrus, Vicia, Lens*
	R. meloloti		*Melilotus, Mediago Triagonella*
	R. loti		*Lotus, Lupinus, Anthyllis, Cicer, Ornithopus*
Bradyrhizobium	*B. japonicum*		*Glycine, Macroptilium*
	B. sp.		*Vigna, Lotus, Lupinus, Cicer*

Nodulation problems attributed to the rhizobial symbiont may be due to the presence or the absence of appropriate strains, low population numbers, low infectivity or infectiveness, poor survival in the soil or competition amongst strains of rhizobia. Legume inoculation is a way of assuring that the strain of *Rhizobium* appropriate for the cultivar being seeded is present in the soil at the proper time and in numbers sufficient to assure a quick and effective infection and subsequent nitrogen fixation.

Taxonomy of chickpea rhizobium

Previous classifications of the root nodule bacteria were based largely on the cross-inoculation group concept, with the assumption that those leguminous plants falling within a particular infection group were nodulated by one particular species of nodule bacteria. This classification gradually lost credibility from a basic point of view with repeated evidence of anomalous cross-infection among the different plant groups. Currently, the classification takes into account newer information based on techniques designed to examine larger portions of the bacterial genome (Jordan, 1984). Root-nodule bacteria are now separated into two genera (Jordan, 1984): *Rhizobium* which are fast growing bacteria and *Bradyrhizobium* which are slow growers (Table 1).

Originally, it was thought that root-nodule bacteria appropriate to chickpea belonged to *Rhizobium leguminosarum* (Simon, 1914), but this was shown to be false by Fred *et al.* (1932), and since the work of Gaur and Sen (1979) chickpea rhizobia have been considered highly specific. These last workers have examined seventy one-strains of root nodule bacteria of *Cicer arietinum* for nodulation on eighty-seven species of legumes. These species represent all the known cross-inoculation groups and were selected from various tribes and genera of families *Fabaceae* and *Mimosaceae*. Gaur and Sen (1979) have observed that all the strains of *Cicer-Rhizobium* nodulated their own host *C. arietinum,* but none could nodulate 85 of the other 87 species of legumes. Only 18 strains could form nodules on the two remaining species, *Sesbania bispinosa* and *S. sesban* for which the nodules were moderately effective and the re-isolates from them nodulated the original host. In reciprocal cross-inoculation tests, only one of the 287 strains of *Rhizobium* from 52 species of legumes nodulated *C. arietinum.*

Consequently, the placing of *Cicer* under "pea group" as proposed earlier is not justified and *Cicer* and its root nodule bacteria should be retained in a distinct cross-inoculation group.

Other results obtained with the characterization of chickpea *Rhizobium* by using immunofluorescence, immunodiffusion and antibiotic resistance have shown the antigenic uniqueness of the chickpea rhizobia and their lack of serological crossreaction with any other *Rhizobium* species, especially with those in *R. leguminosarum* where they had previously been classified.

More recently, Ruiz-Argüeso *et al.* (1988) have examined twenty seven strains of rhizobia nodulating chickpea and although they have found strains with fast and slow growth rates the cultural properties examined do not support the separation of chickpea rhizobia into two distinct groups of the classical fast and slow growing types of root nodule bacteria. It seems that the genetic variability of chickpea root nodule bacteria is rather high and more work is needed to precisely locate tha taxonomic position of this bacteria.

Nodule initiation

Before infection via a root hair, rhizobia bind to the host root and elicit root hair curling. The host cell wall is degraded in a pocket formed by the curled root hair, and the bacteria invade the cell through the degraded portion of the wall (Callaham and Torrey, 1981). Plant cell wall material deposited around the bacteria forms the infection thread. Meanwhile, meristematic activity induced in the root cortex gives rise to the cells that will form the nodule. The infection thread grows into many of these new cells, and the rhizobia are then released into the cytoplasm, surrounded by a peribacterial membrane. Bacteria assume the bacteroid state, differentiation of the plant cells occurs, and nitrogenase activity appears.

Nodule initiation and development depends on the expression of host and microsymbiont genes. Variations in both host and bacterial genomes may affect the sequence of nodule development and the expression of the genes involved in nitrogenase activity and regulation. In some cases root hairs and nodules occur only where lateral roots emerge (Dongre *et. al.*, 1985). Concerning chickpea, little is known of the infection process.

Rhizobium ecology

In the Mediterranean area, a substantial part of the year passes with little or no rainfall, and in the absence of irrigation, agricultural soils dry out. In the absence of the host plant, this desiccation has been recognized for a long time as a potential stress on rhizobial survival. This stress is very often associated with high temperatures.The mechanism relating soil water loss to the numbers of rhizobia surviving desiccation is just beginning to come to light and a review concerning the survival of *Rhizobium* in soil has been proposed by Lowendorf (1980).

Physical and chemical stresses influence numbers and persistance of rhizobia. The effect of low pH on the survival of *Rhizobium* is of considerable importance and critical pH value are found for the different *Rhizobium* species. For instance, at pH 5,5 and below, the abundance of *R. meliloti* is rather low and similar results concerning chickpea rhizobia have been presented (Arsac and Cleyet-Marel, 1986). On the other hand, Graham and Parker (1964) showed that sensitivity of a given species of *Rhizobium* to pH in culture varied up to 1 pH unit according to individual strain.

With movement of winter-planted chickpea into drier areas of the Mediterranean region, it is expected that locations previously not planted with chickpea will be utilized. Introduction of cold tolerant, ascochyta blight resistant lines into new, drier production areas has, in early trials, been accompanied by nodulation deficiency in areas of Algeria, Morocco and Syria. In these new areas, inoculation may be necessary if the benefit of winter planting is to be realized; evaluation of inoculation requirement is therefore an essential component of winter- sown chickpea introduction.

Generally, traditional chickpea growing areas contain a high population of effective rhizobia (>10000/g soil), and little difference in yield will be observed between plants receiving 100 kg N/ha and those dependent on fixed nitrogen. In contrast, soils in new production areas are less likely than traditional chickpea areas to contain populations of the *Cicer*-specific rhizobia, and may show dramatic yield increases when plants are inoculated or nitrogen fertilized. Absence of nodules or nitrogen deficiency symptoms at flowering in unfertilized plants are indications of possible rhizobia absence or ineffectiveness. In this case, follow up in the form of an inoculum response trial using 'best' selected strains is a direct method to determine the role rhizobia play in the deficiency.

Evaluation of superior strains of rhizobium

Greenhouse experiments

The evaluation of rhizobia strains involves the assessment of the relative nitrogen fixation of the particular strains. Experiments are conducted in the greenhouse to quantify the variation in nitrogen fixing ability. Total nitrogen is considered the best measure of the effectiveness of the rhizobial strains, however both plant color and plant dry weight are significantly correlated with total nitrogen (Fig. 1).

Because of the highly specific rhizobial requirements of chickpea cultivars, environmental variables and differences in naturalized rhizobial populations may affect competition of introduced strains with native rhizobia for nodulation (Khurana and Dudeja, 1982). This implies that strain selection program, which may use superior strains from institutions with active selection programs (e.g. ICARDA or INRA) as well as locally selected strains, be conducted at some stage in soil before testing in widespread inoculum response field trials. Selected strains should have the additional capacity to survive in these soils, so that effective nodulation of following chickpea crops by the introduced strain is assured. Crop yield and nitrogen content are adequate measurements of inoculation response. Strain invasiveness (competitiveness) and survival in soils can be evaluated by determining nodule occupancy in serological tests using ELISA (Arsac and Cleyet- Marel, 1986), a simple colorimetric prodedure that can be incorporated into kit form.

Field experiments

Response of nine chickpea cultivars to inoculation and application of 120 kg N/ha in a soil of moderately low N fertility containing a large effective native population of rhizobia at Tel Hadya, Syria is shown in Fig. 2. Yield increases of 10-20% across cultivars with strain CP-39 over the uninoculated control were obtained in this field study after testing for effectiveness and competitiveness in soil greenhouse studies. Cultivars yields are generally not responsive to nitrogen fertilization

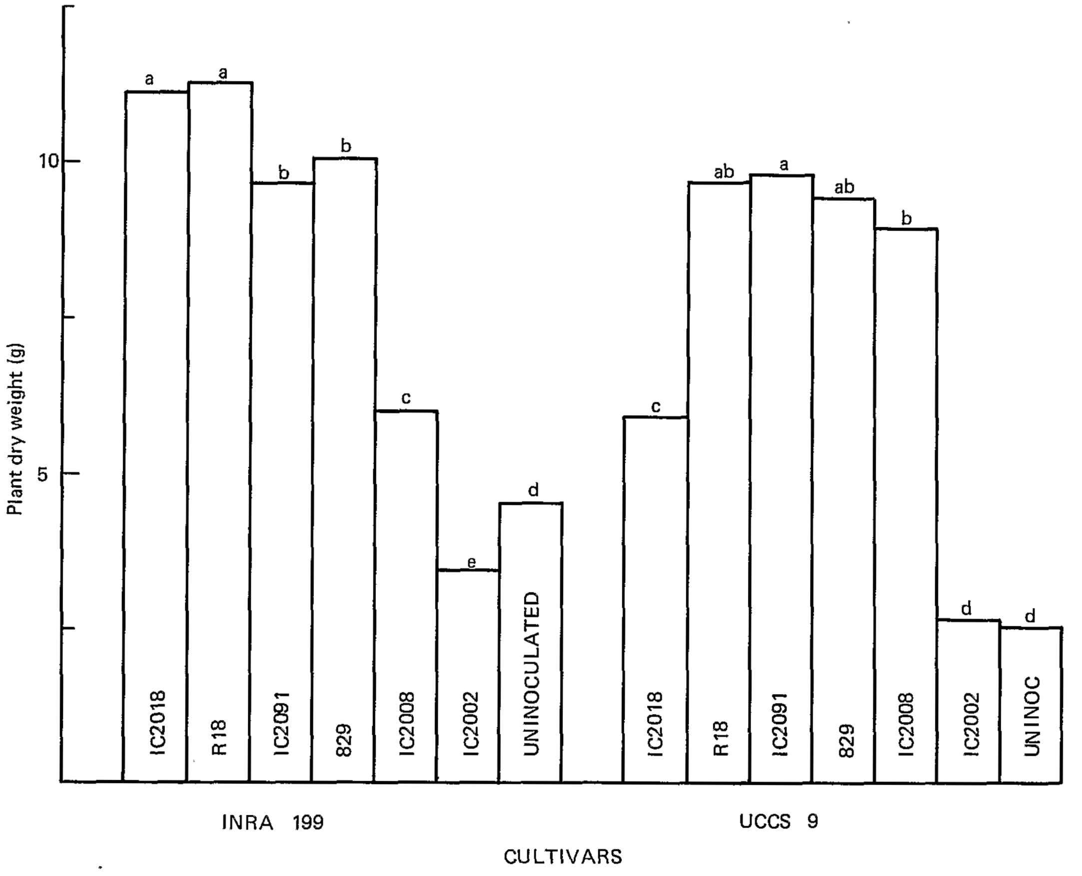

Fig. 1. Nitrogen fixing ability of 6 strains (bars) of chickpea rhizobia. Plants were grown in greenhouse hydroponic culture with nitrogen-free nutrient solution.

where established native rhizobial populations exist, but exceptions, such as FLIP 85-74, appear better able to utilize soil or applied nitrogen.

Benefits of inoculation, however, become more confounded when effects of different strains on yield in a range of cultivars are considered. For example, strain CP-36, which increased seed yield by 30% over that produced by native strains in cultivar ILC 195, depressed yields of ILC 482 and FLIP 85-82 by 17 and 20%, respectively, in a single season experiment at ICARDA (Fig. 3). This strain was selected as highly effective in terms of N-fixing ability on these cultivars in aseptic greenhouse gravel culture. This deleterious effect on yield in soils containing resident rhizobial populations is not uncommon, and illustrates the importance of careful testing prior to an inoculation program. In a mixed-strain inoculation, infection by a less effective strain can counter or dilute effects of more effective strain because of cultivar-specific responses (Rennie and Dubetz, 1984).

Where available soil N is low (<10 ppm) and native rhizobia are absent or present in low numbers (<100 g soil), inoculation with selected strains often boosts yields in excess of 50% (Rupela and Saxena, 1987; Singleton and Tavares, 1986). In soils where native strains are present or soil nitrogen is high (>50 ppm), interactions between chickpea cultivars, soil N and rhizobial strains tend to complicate inoculation studies.

In another series of trials, inoculation with strain IC 2091 was performed in four different soils: Lucca (pH = 5.58, C/N ratio = 9.74), Decima (pH = 7.02, C/N = 9.91), Metaponto (pH = 7.9, C/N = 6.25) Caltagirone (pH = 7.98, C/N = 9.74) and tested with five chickpea cultivars. The seeds were inoculated before sowing with 10^6 rhizobia/seed. The noninoculated plants showed nodules in Lucca, Caltagirone and Metaponto and very few in Decima. Inoculation significantly increased nodule number for the five cultivars analyzed in Decima, Metaponto and Caltagirone but dry weight was signifi-

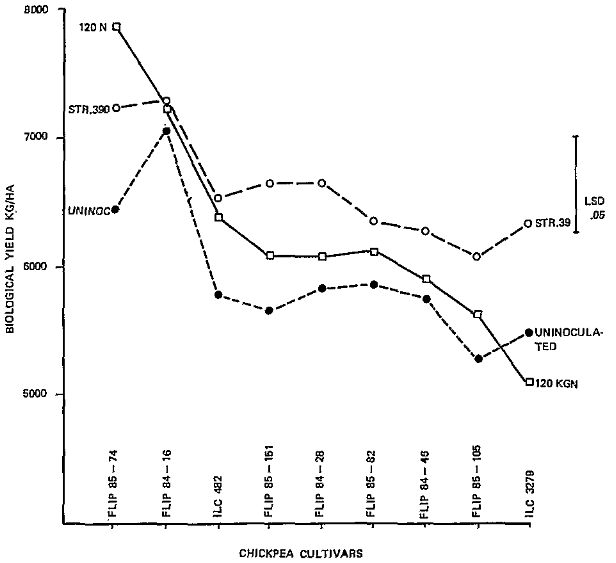

Fig. 2. Effect of mode N nutrition and strain on biological yields of nine chickpea cultivars. Tel Hadya, 1987-88.

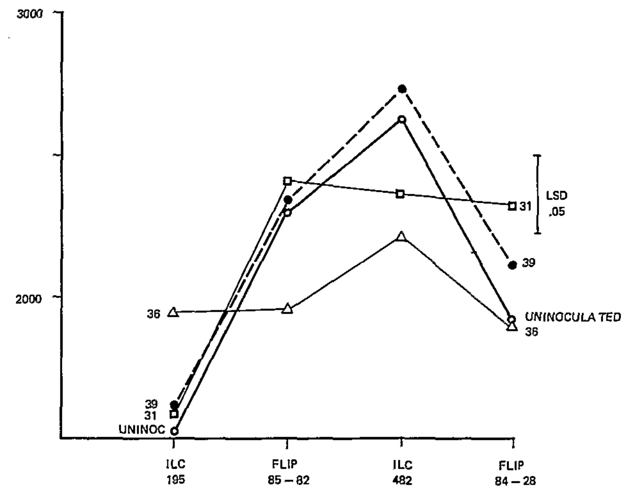

Fig. 3. Effect of *Rhizobium* strain on grain yield of four chickpea cultivars. Tel Hadya, 1987-88.

cantly increased only in Decima (Table 2). The results of inoculation on grain yield at harvest are reported in Table 3 and show higher productivity, with a constantly significant difference, in Decima. It seems that the nodules formed by the indigenous strains in Metaponto, even in lower number, have a non negligable mass and a good nitrogen fixing potential. The unfavourable climate affected plant growth in Caltagirone while the low PH in Lucca affected the establishment of the symbiosis, as has been previously observed (Arsac and Cleyet-Marel, 1986).

Table 2. Effect of inoculation with IC 2091 strain of *Rhizobium* sp. (*Cicer arietinum*) on nodulation of five cultivars of chickpea in different soils. numbers followed by the same letter do not differ significantly (P=0.05) within a given column.

Cultivars		Average of nodule/plant							
		lucca		decima		metaponto		caltagirone	
		No.	Dry w. (g)	No.	Dry w. (g)	No.	Dry w. (g)	No.	Dry w. (g)
Calia	I	1.77	0.02 C	5.93 A	0.17 A	22.50 A	0.85	12.03 A	0.43 AB
	U	2.00	0.06 BC	0.13 B	0.00 B	4.17 B	0.58	1.23 B	0.11 C
Principe	I	2.47	0.04 BC	7.81 A	0.14 AB	24.47 A	0.68	12.87 A	0.49 A
	U	2.53	0.06 AC	0.07 B	0.02 B	7.97 B	0.90	1.87 B	0.25 BD
ILC 482	I	3.50	0.08 AB	10.04 A	0.16 A	17.87 A	1.08	12.87 A	0.44 AB
	U	2.60	0.05 BC	0.10 B	0.00 B	5.73 B	0.76	2.41 B	0.24 BD
ILC 237	I	3.73	0.06 AC	6.23 A	0.10 AB	20.57 A	0.91	12.67 A	0.49 A
	U	3.13	0.06 AC	0.20 B	0.01 B	4.97 B	0.51	2.40 B	0.24 BD
INRA 199	I	2.60	0.12 A	7.10 A	0.06 AB	19.80 A	0.70	13.53 A	0.40 AC
	U	1.63	0.04 BC	0.40 B	0.09 AB	3.37 B	0.31	1.47 B	0.17 CD

I = Inoculated; U = Uninoculated

Table 3. Effect of inoculation with IC 2091 strain of *Rhizobium* sp. (*Cicer arietinum*) on productivity of five chickpea cultivars in different soils. numbers followed by the same letter do not differ significantly (p = 0.05) within a given column.

Cultivar		Grain yield kg/ha			
		lucca	decima	metaponto	caltagirone
Calia	I	2550 B	4375 B	3412 A	650 C
	U	2187 CD	3787 C	3062 AB	887 BC
Principe	I	3100 A	4387 B	2387 B	1312 A
	U	2050 CE	3050 EF	2275 B	1162 AB
ILC 482	I	2300 C	3512 CD	3100 AB	1150 AB
	U	2300 C	2637 FG	2637 AB	1075 AB
ILC 237	I	2050 DF	6225 A	3000 AB	1037 AB
	U	1825 EF	3837 C	2712 AB	1250 A
INRA 199	I	1950 DF	3212 DE	2175 B	900 BC
	U	1737 F	2775 G	2387 B	700 C

I = Inoculated; U = Uninoculated

Conclusion

Cicer arietinum is the third most widely grown legume in the world and even through rhizobia nodulating chickpeas are thought to be highly host specific, *C. arietinum* and its nodule bacterial should be more carefully studied. Sufficient information, generated from work on several temperate legumes, is available to indicate the tremendous potential of adequate inoculation technology. Site- and region-specific data concerning chickpea rhizobia are often lacking or unfortunately of dubious quality. It is now necessary in the Mediterranean area to provide appropriate information about symbiosis, inoculation and the need for inoculation as support for the development of chickpea crop.

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