

Nodulation and nitrogenase activity of chickpea cultivar INRA 199 inoculated with different strains of *Rhizobium ciceri*

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SUMMARY

The nitrogen-fixing capacity of 12 collection strains of *R. ciceri* in symbiosis with chickpea INRA 199 was estimated in the greenhouse by comparison with plant growth in the absence of mineral nitrogen. The strains were separated into 4 groups of significantly different effectiveness. The magnitude of the energy loss in nodule hydrogen production by these strains was estimated to vary between 0.19 and 0.77 in a controlled environment. No hydrogenase activity was detected in any of the associations. In the field, hydrogen production represented 40 % of the energy supply to nitrogenase activity during the growth cycle. A survey revealed considerable variation in nodulation, and in effectiveness of symbiosis, with the *Rhizobium* strains naturally present in soils where chickpea may be grown in future. In some fields there was no spontaneous nodulation. In the majority of fields, the mean dry weight of nodules per plant was around 200 mg at flowering; maximum field nodulation was 700 mg DW nodule plant⁻¹.

Additional key words : *Nitrogen fixation, Rhizobium, hydrogenase, ecophysiology.*

RÉSUMÉ

Nodulation et activité nitrogénase du pois chiche cultivar INRA 199 inoculé avec différentes souches de Rhizobium ciceri.

La capacité fixatrice d'azote de 12 souches de *R. ciceri* en symbiose avec le pois chiche (cultivar INRA 199) est mesurée en serre en comparant la croissance des plantes en absence d'azote minéral. Les souches sont classées en 4 groupes d'efficacité significativement différentes. En milieu contrôlé le pourcentage d'énergie perdu sous forme de production d'hydrogène par les nodosités varie de 0,19 à 0,77. Aucune activité hydrogénase n'a été détectée sur ces souches.

Au champ la production d'hydrogène représente 40 p. 100 de l'énergie allouée à l'activité nitrogénase durant la vie de la plante. La fixation d'azote et la nodulation du pois chiche par les *Rhizobium* naturellement présents dans le sol varie considérablement. Dans certains des sols étudiés, il n'y a pas eu de nodulation spontanée. Dans la majorité des cas, à la floraison, le poids sec moyen des nodosités par plantes est de 200 mg, le maximum mesuré étant de 700 mg.

Mots clés additionnels : *Fixation d'azote, Rhizobium, hydrogénase, écophysiologie.*

I. INTRODUCTION

Chickpea is one of the major grain legumes mostly grown in the world as a source of proteins for human beings. In Europe it is extensively grown in Turkey and Spain. There is some interest in extending its cultivation to other Mediterranean areas of this continent and in North Africa, since it is resistant to drought and grows in low fertility soils.

Like many other grain legumes, chickpea can fix atmospheric nitrogen in symbiosis with bacteria of the genus *Rhizobium*. The corresponding *Rhizobia* are specific and called *R. ciceri* (VAISHYA & SANORIA, 1972 ;

GAUR & SEN, 1979). They differ in their N₂ fixing capacities and the field yield of chickpea can be increased by seed inoculation with the most efficient strains (OKON *et al.*, 1972 ; PATIL & MEDHANE, 1974 ; CORBIN *et al.*, 1977). The efficiency of the strains may be affected by the host cultivar (GAGENDRAGADKAR & VAISHYA, 1983). These observations prompted us to screen collection strains of *R. ciceri* for their ability to fix nitrogen with the chickpea cultivar INRA 199 that has been selected for the south of France (WERY, 1986).

Acetylene reduction and hydrogen evolution were measured on the nodules obtained in the screening in order to evaluate the magnitude of the energy loss due

to nitrogenase H_2 production which is considered as a major limitation of the N_2 fixation efficiency (EVANS *et al.*, 1981; SALSAC *et al.*, 1984).

In this work, field observations are also presented. H_2 evolution and C_2H_2 reduction were measured on dry land cultivated chickpea. Nodulation with native *Rhizobium* in various soils was studied in order to evaluate the need for inoculation in areas where the plant may be grown in the future.

II. MATERIALS AND METHODS

A. *Rhizobium* strains

The 12 *R. ciceri* strains of the screening assay are presented in table 1, with the geographical location of their initial isolation, when known and the collection from which each strain was obtained. Some of these strains were recently characterized by their serogroup (KINGSLEY & BOHLOOL, 1983; ARSAC & CLEYET-MAREL, 1986). The inoculants were prepared in the yeast extract mannitol medium (VINCENT, 1970). After surface sterilization in calcium hypochlorite 1.3 % and germination on 1 % agar enriched with YEM (Yeast extract mannitol medium) the seeds were inoculated by soaking in the liquid inoculant for 30 min.

TABLE 1
Origin of Strains.
Origine des souches.

Name	Collection	Geographical origin
IC 6	ICRISAT	India
IC 165	ICRISAT	India (Labalpur)
IC 2002	ICRISAT	India
IC 2008	ICRISAT	Great Britain
IC 2018	ICRISAT	Turkey
IC 2091	ICRISAT	Great Britain
USDA 3231	USDA	USA (California)
27 A8	NITRAGIN	Mexico
R 18		Argentina
829	ETSIA	Spain
835	ETSIA	Spain (Caceres)
H 45	IARI	India

— Strains were supplied by P. Dart from ICRISAT, H. Keyser from USDA, J. Burton from NITRAGIN, T. Ruiz-Argueso from ETSIA, M. Soto for R 18.

— Les souches ont été fournies par P. Dart de l'ICRISAT, H. Keyser de l'USDA, J. Burton de NITRAGIN, T. Ruiz-Argueso de l'ETSIA, et M. Soto pour la R 18.

B. Plant cultivation

Chickpea cultivar INRA 199 of desi type was grown in the greenhouse under the conditions described previously for soybean (KALIA & DREVON, 1985). These conditions, in which growth and nodulation of chickpea were quite satisfactory, consisted of a one liter liquid nutrient root medium, removed every week, and complemented in the meanwhile with distilled water. The pH was maintained at neutrality with calcium carbonate (0.7 % weight · weight⁻¹) and air was

bubbled permanently into the medium at a flow rate of 400 ml · min⁻¹. During the first three weeks of cultivation, the plants received 1 mM urea; after this, the nitrogen was applied only in the N-fed control treatment.

C. H_2 evolution and C_2H_2 reduction assays

Nitrogenase activity was monitored by the acetylene-reducing activity (ARA) method (HARDY *et al.*, 1968). Since acetylene has been shown to inhibit nitrogenase activity in some legumes (MINCHIN *et al.*, 1983), time course assays were performed on the INRA 199—*R. ciceri* symbiosis with an open-flow device (DREVON *et al.*, 1988). There was no detectable inhibition of C_2H_2 reduction by acetylene. This permitted a measure of ARA on intact plant in closed vessels according to a procedure described earlier for soybean (KALIA & DREVON, 1985).

Assay of hydrogen evolution on intact plants gave us erratic results. Consequently, H_2 evolution was assayed on excised nodulated roots (DREVON *et al.*, 1983). ARA was measured in similar conditions to compute the apparent relative efficiency (aRE) according to SCHUBERT & EVANS (1976): aRE = 1 - (rate of H_2 evolution/rate of C_2H_2 reduction). Hydrogenase activity was checked on a suspension of bacteroids by measuring their aerobic consumption of H_2 (DREVON *et al.*, 1983).

D. Nodulation survey

In the field, ten contiguous plants were harvested at random locations at mid-flowering stage. Dry weights of shoots and nodules, when present, were individually measured after 48 h at 80 °C. Correlation coefficients between these two parameters and regression slopes, when significant, were computed in order to evaluate the effectiveness of the symbiotic interaction.

III. RESULTS

A. Effectiveness of strains

Variations in pod production by chickpea cv. INRA 199 inoculated with the different strains of *R. ciceri* are presented in figure 1. These variations could be attributed to different levels of nitrogen fixation by the inoculants, since the nitrogen nutrition of the plant was the growth-limiting factor, as shown by the lower yield of the non-inoculated non-N-fertilized control treatment. From the statistical analysis on the data in figure 1 with the Newman and Keuls test, the strains were sorted into 4 groups with significantly different ability to fix nitrogen in these experimental conditions:

1. IC 2091, 829, R18 and IC 2018 had the highest N_2 fixing activity. This activity met the N requirement of the plant as well as the weekly 1 mM urea supply in the N control treatment.

2. IC 2008 and USDA 3231 constituted an intermediate group.

3. IC 165, 27 A8, IC 6, 835 and H 45 had a low nitrogen fixation capacity, although their activity was still significant since the yield of plants inoculated with the strains was higher than the yield of the uninoculated plants.

4. IC 2002 was ineffective with chickpea INRA 199.

There was no apparent difference between the speed at which the strains formed nodules. Thus, the first nodules appeared about seven days after inoculation in all treatments.

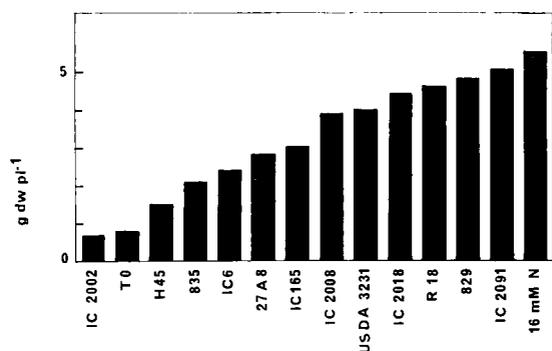


Figure 1
Effect of various strains of *R. ciceri* on chickpea INRA 199 pod yield in the greenhouse. Means of 6 replicates. Plants were grown hydroponically in the greenhouse. The N treatment was 1 mM urea.

Effet de différentes souches de *R. ciceri* sur le poids sec de gousses du pois chiche (Cultivar INRA 199) cultivé en serre. Les valeurs sont la moyenne de 6 répétitions. Les plantes ont été cultivées en serre en milieu hydroponique. Le traitement azoté était de 1 mM d'urée.

B. Nitrogenase activity

The nitrogenase activity of the above four types of strains is shown in figure 2. In the most active strains, activity was maximum 40-50 days after sowing, at the stage of early pod filling. Maximum ARA was 10.6 $\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ pl}^{-1}$ which is lower than for soybean in similar experimental conditions (KALIA & DREVON, 1985). By contrast, the ineffective IC 2002 strain had almost no detectable ARA during the entire growth cycle.

H_2 evolution and ARA by excised nodulated roots were measured for some representative strains of this screening between 40 and 45 days after sowing. The results in table 2 show that the strains having the highest effect on pod yield were the ones having the highest nitrogenase activity at these dates. All strains evolved significant amounts of hydrogen ; the apparent relative efficiency of excised nodules varied between 0.19 and 0.77. No hydrogenase activity was detected on the bacteroids from the nodules formed by the strains of the screening (data not shown).

In the field (fig. 3), nitrogenase activity started later after sowing because the germination process was slowed by the cold temperature of the 1984 month of March. Nitrogenase activity increased regularly during the following 30 days but stopped abruptly due to lack

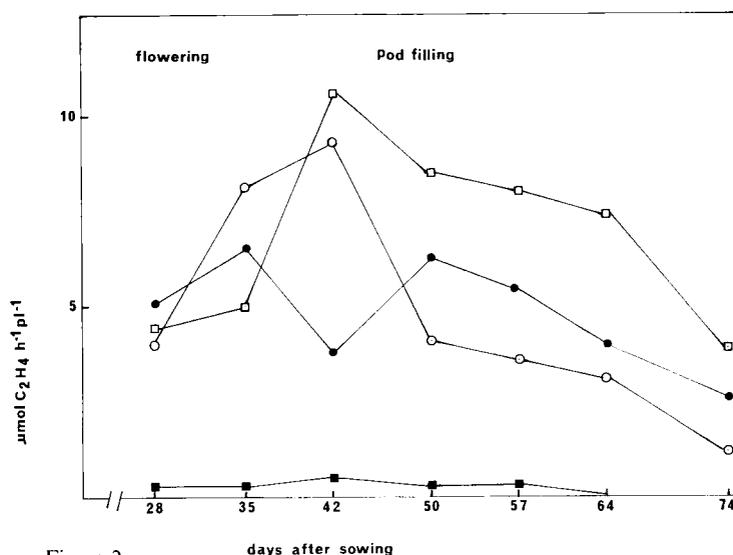


Figure 2
Acetylene-reducing activity of chickpea INRA 199 over its growth cycle in the greenhouse. Means of 4 replicates. *R. ciceri* strains were : -●- IC 6 ; -○- R 18 ; -□- USDA 3231 ; -■- IC 2002.

Mesure de l'activité réductrice d'acétylène du pois chiche (cultivar INRA 199) pendant la croissance des plantes en serre. Les souches de *R. ciceri* sont : -●- IC 6 ; -○- R 18 ; -□- USDA 3231 ; -■- IC 2002. Les valeurs sont la moyenne de 4 répétitions.

of water after this date. Similar drought limitation of chickpea nitrogen fixation was observed in 1983 (COMERE, personal communication). Measurements of hydrogen evolution show that during the period of fixation, about 40 % of the energy supplied to nitrogenase activity was lost in hydrogen production (fig. 3).

Winter-sown chickpea may have a higher nitrogen fixation. Indeed, their nodule fresh weight was significantly higher at a similar date than for spring-sown chickpea : on June 15th, it was $8.0 \pm 2.2 \text{ g fw nodules}$

TABLE 2

Nitrogenase activity (C_2H_2 reduction and H_2 evolution) and apparent relative efficiency of some representative strains from the screening. Activité nitrogénase (réduction de C_2H_2 et émission de H_2) et efficacité relative apparente de souches représentatives du criblage.

Strains	$\mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ pl}^{-1}$	$\mu\text{mol H}_2 \text{ h}^{-1} \text{ pl}^{-1}$	aRE
IC 2002	0.21	0.17	0.19
H 45	1.61	0.58	0.63
835	5.10	1.40	0.73
IC 6	3.70	1.12	0.70
27 A8	6.00	1.80	0.70
IC 165	7.18	1.64	0.78
IC 2008	5.83	2.25	0.61
USDA 3231	10.60	3.79	0.63
IC 2018	15.25	3.50	0.77
R 18	8.30	1.82	0.78
829	9.20	3.50	0.62
IC 2091	9.70	4.20	0.57

Means of 4 replicates. Plants were 40-45 days old during the assay. Lowest significant differences are $4.25 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ pl}^{-1}$, $1.21 \mu\text{mol H}_2 \text{ h}^{-1} \text{ pl}^{-1}$ and 0.07 for aRE.

Les données sont les moyennes de 4 répétitions. Les plantes étaient âgées de 40-45 jours au moment de la mesure. Les plus petites différences significatives sont de $4,25 \mu\text{mol C}_2\text{H}_4 \text{ h}^{-1} \text{ pl}^{-1}$, $1,21 \mu\text{mol H}_2 \text{ h}^{-1} \text{ pl}^{-1}$ et 0,07 pour l'ERa.

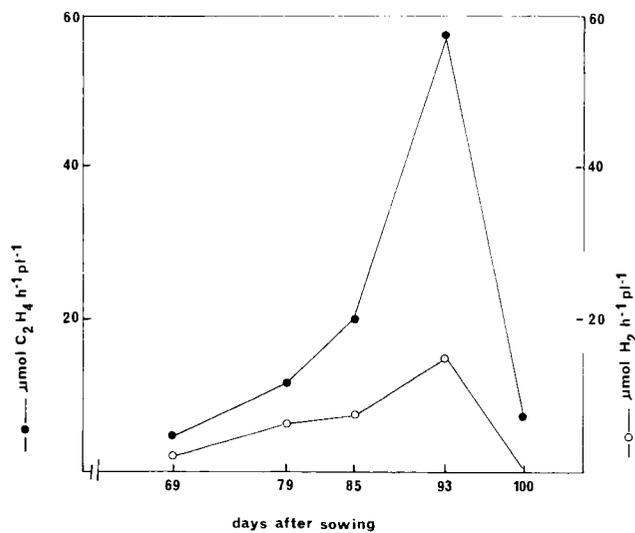


Figure 3

Acetylene-reducing activity and hydrogen evolution of chickpea cv. INRA 199 in the field. Means of 8 replicates. Strains of Rhizobium nodulating chickpea were found in the soil.

Evolution de l'activité réductrice d'acétylène et de l'hydrogène du pois chiche au champ.

Les valeurs sont la moyenne de 8 répétitions. Le pois chiche a été nodulé par des souches de Rhizobium naturellement présentes dans le sol.

pl⁻¹ on winter-sown plants compared with to 2.5 ± 1.2 g fw nodules pl⁻¹ on spring-sown chickpea. The comparison of ARA between winter and spring sown plants would be of major interest.

C. Nodulation survey

The nodulation and shoot dry weights of plants harvested at the flowering stage in 20 fields of the Mediterranean area are shown in table 3. Nodule mass varied considerably. In the majority of the fields, it ranged between 90 and 300 mg dw pl⁻¹. In three locations it was higher than 500 mg dw pl⁻¹. Fields 17, 18 and 20 had no or insignificant nodule mass: either specific effective strains were absent in these fields, or nodulation was strongly inhibited by some pedoclimatic factor.

The mean number of nodules per plant varied from 0 to 52. It was not significantly related to mean nodule mass, some plants having a few big nodules (see field 7), others having numerous small nodules (see field 10). Field 18 showed up as a case of numerous but ineffective nodulation related to a very limited mass of aerial parts.

Plant growth also differed in the various fields. In the overall survey, it did not correlate with mass or number of nodules per plant. This absence of correlation suggests that nitrogen fixation was not the major limiting growth factor of field-grown chickpea, at least in some locations of the survey. This can be illustrated by the comparison between fields 4 and 5 which had similar nodule mass, although shoot dry weight was almost

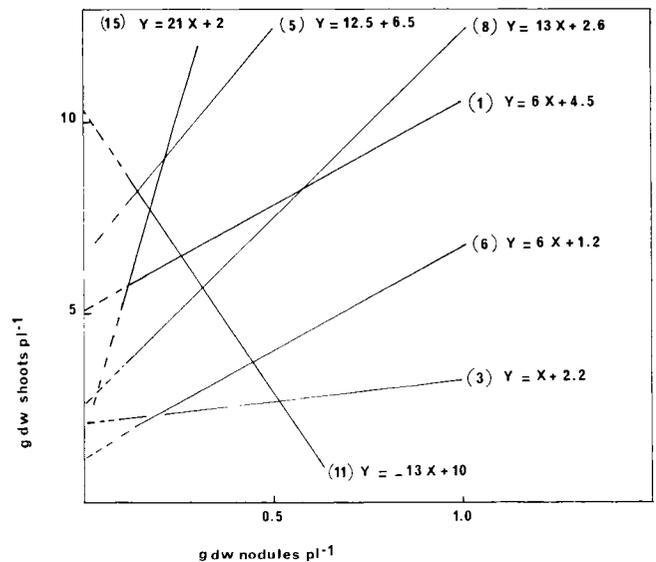


Figure 4

Linear regressions between shoot and nodule weight in survey fields where the two parameters were significantly correlated.

Les régressions linéaires entre le poids sec des parties aériennes et les nodosités dans l'enquête au champ sont corrélées de façon significative.

three times higher in field 5; some environmental factor may have limited the plant response to nodulation in field 4. However an alternate interpretation would be that the nodule-specific activity of the native strains varied from one location to another.

In some locations the soil might have been the major source of N inhibiting nodulation. Fields 7 and 11, in which nodule numbers were low and plant growth was high, could be such cases.

In some fields of the survey, shoot and nodule dry weights were significantly correlated (fig. 4). In the case of soil N limitation, such a correlation indicates that nitrogen fixation was the major limiting growth factor. The intercept of the curve on the shoot dry weight axis may correspond to plant growth in the absence of nodulation and therefore to soil N contribution. The slope could be an evaluation of nodulation efficiency, a steep slope corresponding to highly efficient symbiosis. Thus a similar increase of nodule mass could correspond to a faster growth of plants in the case of a steep slope than in the case of a low slope. However the steepest slopes were not necessarily associated with the highest mean shoot dry weights: see for example field 8 compared to field 1. Soil nitrogen, native strain effectiveness and pedoclimatic factors might be the cause of these discrepancies. Nitrogen fixation would be low either because of poor effectiveness of the symbiosis or because of limitation of its capacity by adverse environmental factors. Some confirmation of this interpretation was found in field 11, where low nodulation presumably due to high soil N content was associated with a negative slope and consequently ineffective nodulation: thus nitrate assimilation is known to inhibit not only nodulation, but also nitrogenase activity (GIBSON, 1976).

TABLE 3

Growth and nodulation of plants harvested in various chickpea fields in the south of France.
Croissance et nodulation des plantes récoltées dans divers champs de pois chiche dans le Sud de la France.

Location	Nodule pl ⁻¹	g DW nod pl ⁻¹	g DW shoot pl ⁻¹
1. La Valette (Hérault)	29 ± 16	0.70 ± 0.60	9.02 ± 5.90
2. Montpellier (Hérault)	52 ± 13	0.68 ± 0.50	10.40 ± 3.60
3. Gareoult (Var)	13 ± 7	0.50 ± 0.50	2.80 ± 0.80
4. Gareoult (Var)	15 ± 10	0.34 ± 0.34	3.30 ± 0.78
5. Digne (Haute-Provence)	21 ± 10	0.32 ± 0.20	9.77 ± 4.43
6. Gareoult (Var)	34 ± 22	0.31 ± 0.21	3.48 ± 0.75
7. Digne (Haute-Provence)	6 ± 3	0.26 ± 0.10	6.50 ± 2.61
8. Carmeiane (Haute-Provence)	36 ± 17	0.22 ± 0.07	5.60 ± 1.30
9. Les Mées (Haute-Provence)	37 ± 12	0.20 ± 0.08	3.32 ± 1.41
10. Puyricard (Bouches-du-Rhône)	40 ± 14	0.17 ± 0.05	1.97 ± 0.71
11. Mauguio (Hérault)	3 ± 6	0.15 ± 0.2	8.30 ± 8.10
12. Valensole (Haute-Provence)	12 ± 5	0.15 ± 0.07	7.40 ± 2.57
13. Carmeiane (Haute-Provence)	12 ± 5	0.15 ± 0.07	7.40 ± 2.57
14. Figuière (Haute-Provence)	17 ± 11	0.12 ± 0.0	1.86 ± 0.65
15. Thoard (Haute-Provence)	5 ± 5	0.10 ± 0.09	5.20 ± 2.97
16. Gotheron (Drôme)	4 ± 4	0.10 ± 0.14	6.50 ± 4.30
17. Valensole (Haute-Provence)	3 ± 7	0.09 ± 0.1	4.52 ± 1.66
18. Puyricard (Bouche-du-Rhône)	17 ± 10	0.03 ± 0.02	0.33 ± 0.08
19. Carmeiane (Haute-Provence)	6 ± 5	0.02 ± 0.01	0.97 ± 0.22
20. Digne (Haute-Provence)	0	0	1.76 ± 0.41

Means ± standard deviations of 10 replicates; plants were harvested at the mid flowering stage.

Les données sont les moyennes ± écart type 10 répétitions; les plantes ont été récoltées au stade mi-floraison.

IV. DISCUSSION

The collection strains of *R. ciceri* varied in their capacity to fix nitrogen with chickpea cv. INRA 199. Strain H45, known as an efficient strain (GAGENDRA-GADKAR & VAISHYA, 1983), had low nitrogen fixation in this screening, which suggests a negative effect of the macrosymbiont on the expression of this strain's capacity to fix nitrogen. However INRA 199 seemed to be able to exploit fully the nitrogen fixation process, since its yield in the presence of the best fixing strains in this assay was similar to that obtained with combined nitrogen. Field inoculation assays are needed to check the ranking of strains in agricultural conditions and evaluate their effect on yield in fields where there is no *R. ciceri*, and in fields where the introduced strain will have to compete with native *Rhizobia* which are able to nodulate chickpea.

The relative amounts of hydrogen evolved by these strains were of the same order of magnitude (MINGUEZ & RUIZ-ARGUESO, 1980) or lower (SINDHU *et al.*, 1986) than those described previously. They represent a loss of 25 to 60 % of the energy required for nitrogenase activity. However *in situ* measurement of H₂ evolution is needed to check that the high production of H₂ by excised nodulated roots was not an artefact. Thus excision is known to modify the oxygen supply of the bacteroids (SHEEHY *et al.*, 1985), which is a determinant factor of the apparent relative efficiency of soybean

(DREVON *et al.*, 1982). The absence of hydrogenase activity in the *R. ciceri* strains of this screening, in addition to previous similar observations in other isolates (MINGUEZ & RUIZ-ARGUESO, 1980; SINDHU *et al.*, 1986) suggests that the frequency of Hup⁺ (hydrogenase uptake positive) strains is low in the symbiosis with chickpea, which is not the case in all *Rhizobium* species (EISBRENNER & EVANS, 1982).

The field survey showed that *R. ciceri* was absent in some locations of possible cultivation of chickpea in France. Inoculation might be necessary although the observed absent or poor nodulation could be due to some adverse environmental factor. More information on soil and climate would be necessary for a more detailed analysis in a further survey. It would help to improve chickpea nitrogen fixation in its area of extension and to determine where inoculation would be beneficial.

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