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Original article

Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Moroccan soils

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Abstract – Phenotypic characteristics of fifty-six rhizobia strains isolated from root nodules of two chickpea (*Cicer arietinum* L.) cultivars, growing in soils collected from different areas of Morocco, were studied. Tolerance to salinity, high temperatures, acid and alkaline pHs, heavy metals and to antibiotics as well as symbiotic and cultural characteristics allowed the description of a wide physiological diversity among tested isolates. Numerical analysis of the phenotypic characteristics showed that, below the boundary level of 82% average similarity, isolates fell into at least five distinct groups. A number of potential isolates have been identified for inoculation trials. They were effective and able to grow at pH ranging from 5 to 8, tolerated salt concentration (1–1.5%) and grew at a maximum temperature between 30 and 35 °C.

biodiversity / *Cicer arietinum* / phenotypic characterization / *Rhizobium*

Résumé – Caractérisation phénotypique des rhizobia isolés du pois chiche (*Cicer arietinum*) cultivé sur les sols marocains. Cinquante six souches de rhizobium nodulant deux cultivars de pois chiche (*Cicer arietinum* L.) et isolées de différentes régions du Maroc, ont été caractérisées sur le plan phénotypique. L'analyse de leur tolérance à la salinité, aux températures élevées, aux pH acides et alcalins, aux antibiotiques, aux métaux lourds ainsi que leurs caractéristiques symbiotiques et culturales ont permis de mettre en évidence une large diversité physiologique au sein de ces populations de rhizobium nodulant le pois chiche. L'analyse numérique de ces caractéristiques phénotypiques a montré qu'à un niveau de 82 % de similarité, ces souches bactériennes se sont réparties en cinq groupes distincts. Des souches intéressantes pour les essais d'inoculation ont été identifiées. Elles sont efficientes et capables de tolérer des pHs allant de 5 à 8, des concentrations en NaCl de 1 à 1,5 % et des températures maximales comprises entre 30 à 35 °C.

biodiversité / caractérisation phénotypique / pois chiche / *Rhizobium*

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1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important legume food crop in many developing countries, and there are substantial research programmes to improve its yield, disease resistance and nutritional quality. In Morocco, during the last decade, the chickpea crop has been progressively extended from semi-arid to arid areas where edaphoclimatic conditions such as salinity, pH and temperature have adverse effects on the establishment of functional symbiosis [13, 20]. Although inoculation assays with non-native strains of chickpea rhizobia were used to improve the production of this crop, the yield obtained in those regions is currently low. The yield is not only limited by the N_2-fixing potential of the chickpea-rhizobium symbiosis but also the non-adaptability of both symbionts to the osmotic stress prevailing in those areas. Arid soils may contain well-adapted strains to the prevailing extreme environmental stresses such as drought, high soil temperature and pH [14]. Such strains should be isolated and evaluated for use as chickpea inoculants for the large arid areas of Morocco.

Although chickpea nodulating bacteria are highly specific to their host plant [6, 12], in all published data concerning these rhizobia a large diversity has been demonstrated, leading to the description of at least two species [16, 17].

In this context, we have isolated chickpea rhizobial isolates from various soils of the arid and semi-arid areas of Morocco and initiated phenotypic studies in order to examine their biodiversity.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

The fifty-six rhizobial isolates used in this study were isolated from root nodules of young plants of chickpea, *Cicer arietinum* (cultivars PCH34 and PCH37) grown in soil samples collected from arid (200–300 mm rain·year^{-1}) and semi-arid (300–400 mm rain·year^{-1}) regions of Morocco. Some of those soil samples were considered as saline soils since they have a high soluble salts concentration, leading to soil electrical conductivity higher than 4×10^{-3} mhos·cm^{-1}. Pure isolates were maintained at –20 °C in 50% glycerol and Yeast Extract-Mannitol (YEM) was routinely used as complete medium for rhizobium culture [23].

2.2. Growth characteristics

Bacterial growth was assessed on YEM liquid medium incubated in a gyratory shaker at 200 rev·min^{-1}, by measuring the optical density at 600 nm every 2 h. The generation time (GT) was calculated from the logarithmic phase of growth.

2.3. Symbiotic characteristics

To estimate their infectivity and their effectiveness in fixing atmospheric nitrogen, each strain was grown on YEM liquid medium to logarithmic phase (~10^9 cell·ml^{-1}) and inoculated to aseptic germinated seeds of chickpea growing in pots (three seedlings/pot) containing sterilized and nitrogen-free sand. The experiment was statistically laid out with three replications using randomized block design. As control, each block contained two pots (T0 and TN) with uninoculated seedlings. Plants were supplied with distilled water every two days, and they were saturated once a week with a nitrogen-free nutrient solution. Furthermore, TN control received weekly 0.5‰ (w/v) KNO_3 as nitrogen source. Plants were grown in a growth chamber with a 16/8 h light/dark cycle and 28/20 °C day/night temperature. Forty-five days after planting, nodule number and dry weight of nodules and shoots were recorded. These data were statistically analyzed using Fisher's protected LSD (Least Squares Differences) for means comparison.

2.4. Physiological characteristics

All tests, except carbohydrate assimilation, were carried out on YEM agar plates. Petri dishes containing defined medium were subdivided into squares and each square was inoculated with 10 µl of 48 h bacterial YEM broth. After 7 days of incubation at 28 °C, bacterial growth was compared to the controls. Two replicates were included per treatment.

2.4.1. Salt tolerance

It was determined on YEM agar plates containing from 0 to 5% (w/v) NaCl or $CaCl_2$ concentrations.

2.4.2. pH tolerance

Tolerance to extreme pH was tested on YEM agar medium set at different pH values, using the buffers MES

(20 mM) (SIGMA) for pHs ranging between 5.5 and 6.7, and HCl for pHs lower than 5.5; MOPS (20 mM) (SIGMA) for pHs ranging between 6.7 and 7.9 and NaOH for pHs up to 9.5.

2.4.3. Temperature tolerance

Yeast extract-mannitol agar plates were inoculated as described above and incubated at temperatures from 5 to 45 °C.

2.4.4. Intrinsic antibiotic and heavy metal resistance

This intrinsic resistance was determined on solid YEM medium containing the following filter sterilized antibiotics or heavy metals ($\mu g \cdot ml^{-1}$): ampicillin (50), chloramphenicol (10), erythromycin (100), kanamycin (10 and 100), nalidixic acid (50), rifamycin (10), spectinomycin (10), streptomycin (25 and 100) and tetracyclin (20); $AlCl_3$ (250), $CdCl_2$, $2H_2O$ (20), $CoCl_2$ (25), $CuCl_2$, $2H_2O$ (50), $HgCl_2$ (10), $MnCl_2$ (500), $NiSO_4$ (100), $Pb(CH3COO)_2$ (250), and $ZnCl_2$ (50).

2.4.5. Carbohydrate assimilation

Utilization of organic compounds as the sole carbon source was tested by using API galleries (API 50 CH; BioMérieux, France) as described by Kersters [11]. Inocula of the tested isolates were obtained from 48 h YEM agar plates. As some isolates, however, were able to grow in YEM liquid medium from which the mannitol was omitted, inocula were resuspended in the following synthetic medium at 45 °C ($g \cdot l^{-1}$): K_2HPO_4, 0.46; KH_2PO_4, 0.12; $MgSO_4$, 0.20; NaCl, 0.1; $(NH_4)_2SO_4$, 2; yeast nitrogen base, 2 and pure agar, 7. Inoculated galleries were incubated at 28 °C. Results were scored after 1, 2, 4 and 7 days.

2.5. Numerical analysis

A computer cluster analysis of phenotypic variables was carried out using a similarity coefficient and a phenogram was constructed by the unweighed pair group method with the average (UPGMA) clustering method.

3. RESULTS AND DISCUSSION

3.1. Symbiotic characteristics

Tested isolates of chickpea rhizobia showed a large diversity in their capacity to infect the host plant and to fix atmospheric nitrogen. The nodule mean number per plant ranged from 5 for isolate Rch 30b to 62 for isolate Rch 8, being the most infective isolate (Fig. 1). In comparison with TN control which represents the 100% level of shoot dry matter and T0 control which represents 36%, most of the tested isolates showed a dry matter yield higher than T0. Indeed, the relative effectiveness, expressed in percent of TN control, showed that strain Rch126b was the most efficient with 88% dry matter yield while Rch69 was the least efficient with only 43% (Fig. 2). The means comparison showed several overlapping groups. Among isolates included in the same group as TN control, i.e. allowing more than 75% of TN dry matter yield, five isolates (Rch1, 18b, 125b, 126b and 128b) were particularly infective and efficient. These isolates must be taken into consideration for chickpea inoculation under Moroccan edaphoclimatic conditions.

Although some authors [19] showed a significant correlation between the increase of dry matter and the number or the dry weight of nodules, such correlation was not found in our study. It has been shown that the dry matter yield was rather correlated with the nodule leghaemogblobin concentration than with the number or the dry weight of nodules [5].

3.2. Growth characteristics

On the basis of their generation times 22% of the isolates were fast growers (50 min < GT < 3 h), 32% slow growers (3 h < GT ≤ 9 h) and 46% were extra-slow-growing bacteria with a generation time of more than 9 hours (Fig. 3). Distribution of such rhizobia in arid and semi-arid areas was not correlated with the climatic region, i.e. fast, slow and very slow-growing rhizobia could be found in the same soil.

3.3. Physiological characteristics

As shown in Figure 4, the chickpea nodulating rhizobia tested showed a wide diversity in their pH tolerance. From 90 to 100% of the isolates grew in lightly acid and neutral pH. At low pH, some isolates exhibited an

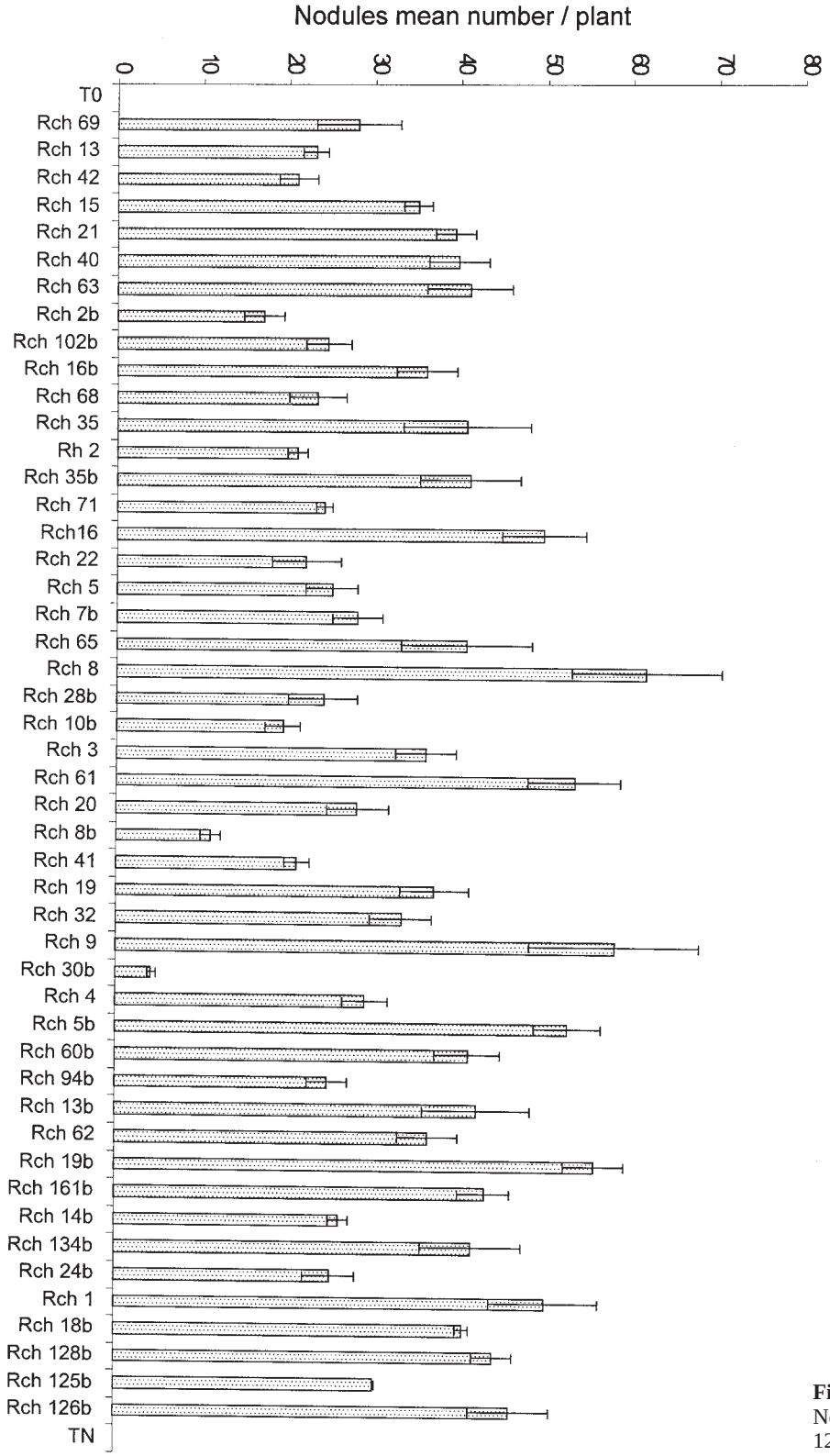

Figure 1. Infectivity of chickpea rhizobia. Nodule numbers are the mean ± SD of 6 to 12 plants randomized in 3 pots.

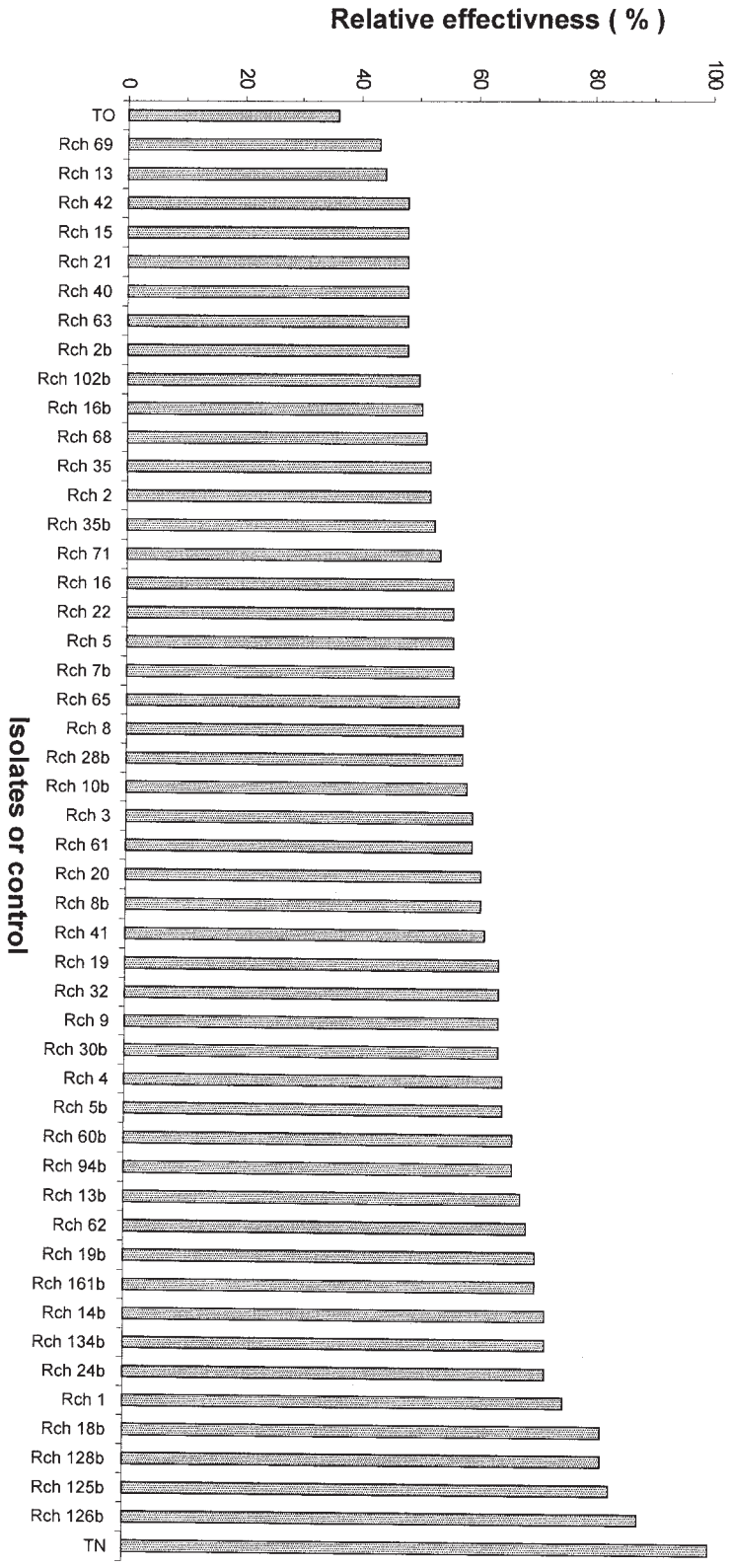

Figure 2. Relative effectiveness (RE) of chickpea rhizobia, determined according the following equation: RE = (DWi / DWTN) × 100; DWi: dry weight of inoculated plant; DWTN: dry weight of plant control (uninoculated but fertilized with KNO_3).

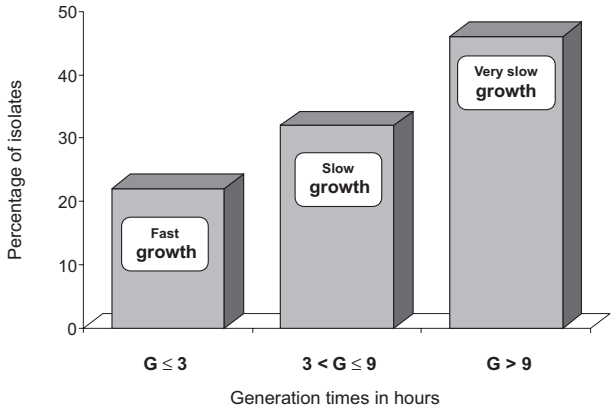

Figure 3. Generation times of tested chickpea rhizobia.

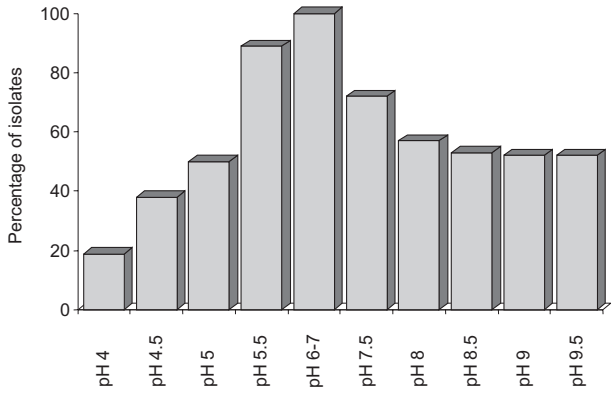

Figure 4. Effect of pH on growth of chickpea rhizobia.

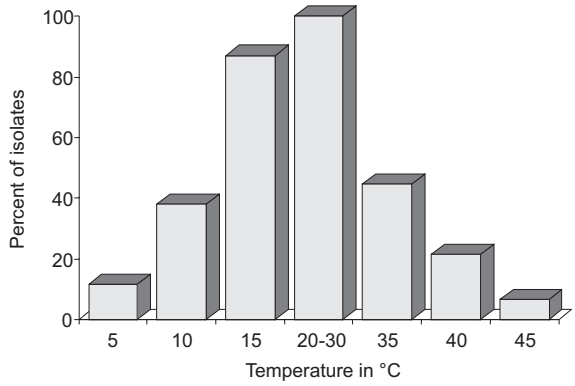

Figure 5. Effect of temperature on growth of chickpea rhizobia.

acido-tolerant character since they grew without restriction in pH 4. Above pH 7.5, from 50 to 70% of the isolates grew in alkaline pHs. As reported already [15], chickpea rhizobia show a neutral and baso-tolerant tendency. This tendency might be related to the basic pH that characterizes most of the origin soils of the tested isolates.

As shown in Figure 5, maximum growth of all tested strains was obtained between 20 and 30 °C. Below and above those values, the percentage of isolates that grew decreased to reach 12% at 5 °C and 7% at 45 °C. It has been reported that slow-growing rhizobia were more thermo-tolerant than fast-growing rhizobia [18]. More than 50% of tested isolates were very slow growers, not able to tolerate more than 42–45 °C. Although isolates that tolerated 45 °C were isolated from a saline arid region with a high summer temperature, no correlation between climatic region and tolerance to low or high temperature was observed.

The data in Figure 6 show that chickpea rhizobia exhibited a wide diversity in their salt tolerance. The salt inhibitory concentrations varied among strains and salt nature. Indeed, tolerance to sodium chloride (NaCl), was found since more than 80% of the tested rhizobia continued to grow with 1% NaCl. However, at higher concentrations, the percentage of tolerant strains decreased rapidly and only three strains showed a moderate growth in 5% NaCl. Conversely, the $CaCl_2$ inhibited all tested isolates at lower concentrations, and above 0.8% none of the isolates grew. It is often believed that saline soils naturally select strains more tolerant to salinity. To verify this hypothesis, the growth of chickpea rhizobia with various salt concentrations was compared (Fig. 7). Indeed, in the presence of 2% NaCl, 90% of isolates from non-saline soils were inhibited, whereas 40% of isolates from saline soils continued to grow. These results confirm that the natural habitat of the strains performs a selection pressure for tolerance to salinity.

The evaluation of intrinsic resistance to antibiotics of chickpea rhizobia showed that most of the tested isolates (65%) exhibited high resistance to kanamycin, nalidixic acid and erythromycin (Fig. 8). In the presence of ampicillin, chloramphenicol, rifamycin, spectinomycin, streptomycin or tetracyclin, only 14 to 25% (according to antibiotic) of isolates were resistant. A comparable behavior was observed with heavy metals (Fig. 9). More than 75 and 82% of isolates showed good tolerance to mercury and manganese, 40 and 46% to copper and cobalt. Other heavy metals were more inhibitory since only about 20% of isolates exhibited an intrinsic resistance to

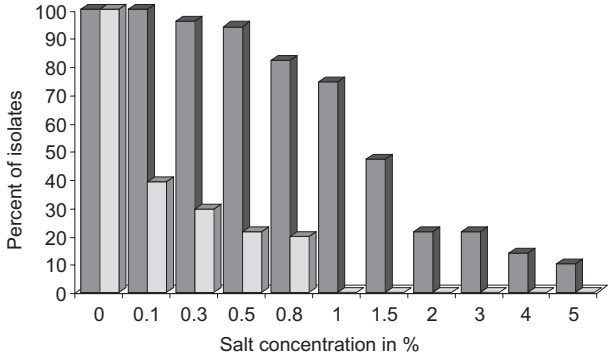

Figure 6. Tolerance of chickpea rhizobia to different concentrations of NaCl (■) and $CaCl_2$ (□).

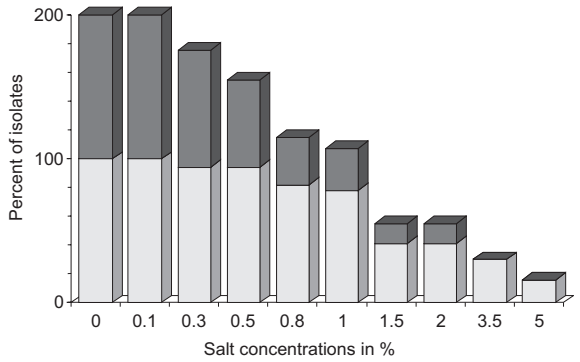

Figure 7. Behavior of two groups of chickpea rhizobia towards (NaCl) according to their origin. Isolates from non-saline soils (■) or from saline soils (□).

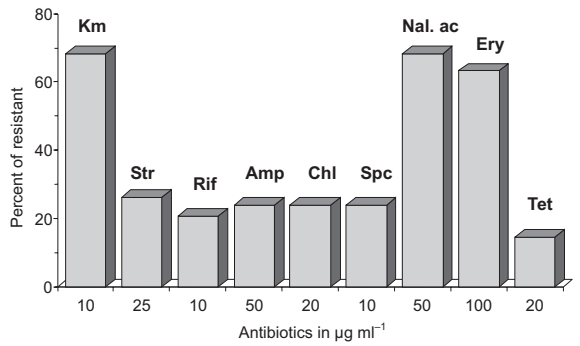

Figure 8. Effect of different antibiotics on growth of chickpea rhizobia.

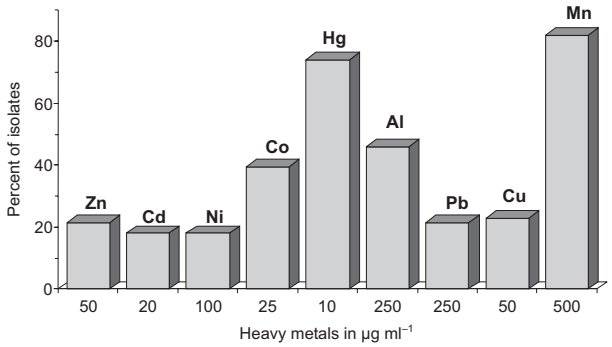

Figure 9. Effect of different heavy metals on growth of chickpea rhizobia.

them. However, the effective concentrations available to these isolates will be lower than those added since metal ions complex with agar and media components [3]. Therefore, the tested rhizobia may be sensitive to much lower concentrations in soils.

Most of the chickpea rhizobia strains were able to catabolize a large variety of carbon substrates (Tab. I). All tested strains grew on glycerol, D-fructose, N-acethyl glucosamine, sorbitol, mannitol, sucrose, trehalose, L-fucose, gluconate, L-arabitol, maltose and cellobiose. None of them utilized L-xylose, glycogen, inulin or α-methyl-D-mannoside. With regard to the remaining carbohydrates, the rhizobial strains exhibited a large diversity. As reported [21, 22], fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. However, our results show the majority of tested chickpea rhizobia were able to use a broad range of carbohydrates though they were very slow growers. Such results are in agreement with those of Nour et al. [15] with chickpea rhizobia.

For temperature, pH and salinity, the 5 efficient strains (Rch1, 18b, 125b, 126b and 128b) identified above, were able to grow between 30 and 35 °C, pH 5 and 8 and to tolerate NaCl concentrations up to 1.5%. Thus, they could be good candidates for chickpea inoculation under Moroccan edaphoclimatic conditions. These data are the basis for strain improvement and cross-inoculation experiments with different species or varieties when searching for well adapted and compatible partners.

The phenotypic studies are necessary for the characterization and selection of strains adapted to marginal

Table I. Carbohydrate utilization by chickpea rhizobia.

Carbohydrate	% of isolates growing on the carbohydrate
Glycerol, D-fructose, N-acethyl glucosamine, sorbitol, mannitol, sucrose, trehalose, L-fucose, gluconate, L-arabitol, maltose, cellobiose	100
L-arabinose, inositol, arbutin, ß-gentibiose	98
2-ceto-gluconate, ribose, aesculin	96
Galactose	94
Adonitol	91
D-Arabitol	89
D-Turanose	87
D-Arabinose,	85
Rhamnose, D-lyxose	84
D-Xylose	82
5-Ceto-gluconate, xylitol	77
D-Glucose	75
D-Tagatose	64
D-Raffinose	62
Erythriol, melibiose	45
Dulcitol	37
Salicin, lactose	34
D-Mannose	25
α-Methyl-D-glucoside	16
D-Fucose	12
L-Sorbose	9
Melezitose, ß-methyl-D-xyloside	5
Amygdalin, starch	4
L-Xylose, glycogen, inulin, α-methyl-D-mannoside	0

edaphoclimatic conditions and provide information about their genetic diversity.

3.4. Numerical analysis

Physiological and biochemical studies are the basis for a detailed polyphasic taxonomy, though they cannot be used alone in taxonomic analysis. However, results concerning carbon sources, salinity and maximum growth temperature may give indications about the taxonomic position of the strains.

Numerical analysis of the phenotypic characteristics showed that, below the boundary level of 82% average similarity, the tested rhizobia isolates can be grouped into at least five groups (Fig. 10). Previously, bacteria that are able to establish effective symbiotic relationships with chickpea were considered as a singular group on the basis of the specificity for the plant host [6], the serological and antigenic characteristics [12], the cultural traits and the polymorphism of *nif* HD genes [1]. However, on the basis of the growth rate, they were classified as *R. loti* (fast-growing rhizobia) [4, 7], or as *Bradyrhizobium* sp. (slow growers) [9]. The inclusion of chickpea rhizobia into two different genera based on the wide range of generation times was discussed [2, 10]. Later, based on a polyphasic study, two new genomic species, *Rhizobium ciceri* and *Rhizobium mediterraneum*, were identified [15, 16, 17], then were later transferred to the *Mesorhizobium* genus [8].

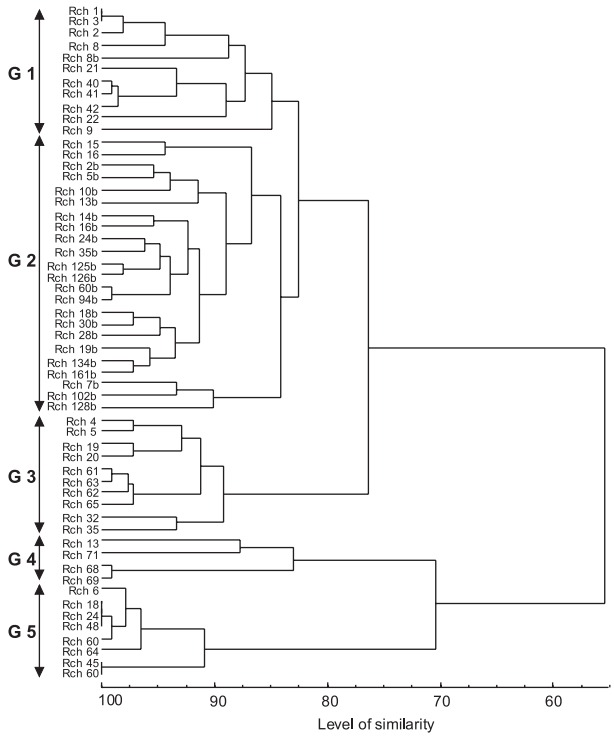

Figure 10. Phenogram highlighting the phenotypic similarity among isolates from chickpea nodules growing in different areas of Morocco.

4. CONCLUSION

The fact that 5 phenotypic groups were identified suggests that several genomic species might exist in Moroccan soils. To verify this suggestion, we need to complete this study using molecular techniques such as REP/PCR or RFLP/PCR, sequencing of 16S rDNA genes and DNA/DNA hybridization.

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REFERENCES

[1] Cadahia E., Leyvar A., Ruiz-Argueso T., Indigenous plasmids and cultural characteristic of rhizobia nodulating chickpeas (*Cicer arietinum* L.), Arch. Microbiol. 146 (1986) 239–244.

[2] Chakrabarti S.K., Mishra A.K., Chakrabartty P.K., DNA homology studies of rhizobia from *Cicer arietinum* L., Can. J. Microbiol. 32 (1986) 524–527.

[3] Chaudri A.M., McGrath S.P., Giller K.E., Metal tolerance of isolates of *Rhizobium leguminosarum* biovar *trifolii* from soil contaminated by past applications of sewage sludge, Soil Biol. Biochem. 24 (1992) 83–88.

[4] Crow V.L., Jarvis B.D.W., Greenwood R.M., Deoxyribonucleic acid homologies among acid producing strains of *Rhizobium*, Int. J. Syst. Bacteriol. 31 (1981) 152–172.

[5] Dudeja S.S, Khurana A.L., Kundu B.S., Effect of *Rhizobium* and phosphomicroorganisms on yield and nutrient uptake in chickpea, Curr. Sci. 50 (1981) 503–505.

[6] Gaur Y.D., Sen A.N., Cross inoculation group specificity in *Cicer Rhizobium* symbiosis, New Phytol. 83 (1979) 745–754.

[7] Jarvis B.D.W., Pankhurst C.E., Patel J.J., *Rhizobium loti*, new species of legume-root nodule bacteria, Int. J. Syst. Bacteriol. 32 (1982) 371–380.

[8] Jarvis B.D.W., van Berkum P., Chen W.X., Nour S.M., Fernandez M.P., Cleyet-Marel J.C., Gillis M., Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium meditrraneum*, and *Rhizobium tianshanense* to *Mezorhizobium* gen. nov., Int. J. Syst. Bacteriol. 47 (1997) 895–898.

[9] Jordan D.C., Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov. a genus of slow-growing root-nodule bacteria from leguminous plants, Int. J. Syst. Bacteriol. 32 (1982) 136–139.

[10] Jordan D.C., Family III *Rhizobiaceae*, in: Krieg N.R., Holt J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, Vol. 1, The Williams, 1984, pp. 234–242.

[11] Kersters K., Hinz K.H., Hertle A., Segers P., Lievens A., Siegmann O., de Ley J., *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds, Int. J. Syst. Bacteriol. 34 (1984) 56–70.

[12] Kingsley M.T., Ben Bohlool B., Characterization of *Rhizobium* sp. *Cicer arietinum* L. by immunofluorescence, immunodiffusion and intrinsic antibiotic resistance, Can. J. Microbiol. 29 (1983) 518–526.

[13] Lauter D.J., Munns D.N., Clarkin K.L., Salt response of chickpea as influenced by N supply, Agron. J. 73 (1981) 961–966.

[14] Mpepereki S., Makonese F., Wollum A.G., Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils, Symbiosis 22 (1997) 275–292.

[15] Nour S.M., Cleyet-Marel J.C., Beck D., Effosse A., Fernandez M.P., Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.), Can. J. Microbiol. 40 (1994a) 345–354.

[16] Nour S.M., Fernandez M.P., Normand P., Cleyet-Marel J.C., *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.), Int. J. Syst. Bacteriol. 44 (1994b) 511–522.

[17] Nour S.M., Cleyet-Marel J.C., Normand P., Fernandez M.P., Genomic heterogeneity of strains nudulating chickpea (*Cicer arietinum* L.) and description of *Rhizobium mediterranium* sp. nov., Int. J. Syst. Bacteriol. 45 (1995) 640–648.

[18] Robert F.M., Molina J.A.E., Schmitdt E.L., Properties of *Rhizobium leguminosarum* isolated from various regions of Morocco, Ann. Microbiol. 133 (1982) 461–470.

[19] Rupela O.P., Dart P.J., Research on symbiotic nitrogen fixation by chickpea at ICRISAT, in: Proceedings of the International Workshop on Chickpea Impovement, 28 Feb.–2 Mar., Hyderabad, A. P., India, 1980.

[20] Singleton P.M., Ben Bohlool B., Effect of salinity on nodule formation by soybean, Plant Physiol. 74 (1984) 72–76.

[21] Stowers M.D., Carbon metabolism in *Rhizobium* species, Annu. Rev. Microbiol. 39 (1985) 89–108.

[22] van Rossum D., Schuurmans F.P., Gillis M., Muyotcha A., van Verseveld H.W., Stotthamer A.H., Boogerd F.C., Genetic and phenotypic analysis of *Bradyrhizobium* strains nodulating Peanut (*Arachis hypogae* L.) roots, Appl. Environ. Microbiol. 61 (1995) 1599–1609.

[23] Vincent J.M., A manual for the Practical Study of Root-nodule bacteria. IBP Handbook No. 15, Blackwell Sci. Publ., Oxford, 1970.