Moroccan Journal of Chemistry
ISSN: 2351-812X
http://revues.imist.ma/?journal=morjchem&page=login
A. khadraji & al. / Mor. J. Chem. 5 N°4 (2017) 722-729

Phosphate solubilization capacity, salinity and drought tolerance of rhizobia nodulating chickpea (Cicer arietinum L.)

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Received 24 May 2017,
Revised 11 July 2017,
Accepted 27 July 2017

Abstract

This study aims to select efficient rhizobia strains nodulating chickpea (*Cicer arietinum L.*) cultivars from the semi-arid zone in Southern Morocco, and evaluated their performance in order to ameliorate the N_2 fixing symbioses of this crop. 68 isolates were obtained from the chickpea nodules, where only 12 strains were selected for their strong potential for nodulation. Many abiotic stress tolerance were assessed for the studied rhizobia such as salinity (0 to 1711 mM), acidity (pH 6.8 to 3.5) and drought (0 to -0.23 MPa PEG_{6000}). Bacterial phosphate-solubilizing ability in NBRIP medium has demonstrated the ability of the studied strains to mobilize significant amounts of P from tricalcium phosphate (TCP), especially the MRP6 strain with a solubilizing index (PSI) of 4.1 and which has also showed high tolerance to salinity (1711 mM), and drought (-0.23 MPa). According to the obtained results, P solubilizing ability was directly correlated to the pH decrease in the medium. The maximum level of orthophosphate was released by MRP13 strains with a value of 1195 mg of P L^{-1} after 5 days of incubation.

Keywords: *Chickpea, rhizobia strains, P-solubilizing, stress tolerance, drought.*

1. Introduction

Chickpea was one of the first grain legumes to be domesticated from its origin in south-eastern Turkey and Syria [1]. Today this crop is grown in at least 33 countries and covers 14 million hectares of the harvested area with a production of more than 13 million tones [2]. This species could provide 144 Kg per Ha per year of N to the soil in association with rhizobia [3]. Several reports have demonstrated the important role of stress tolerant rhizobia strains in the enhancement of legume biomass production through its direct effects on root and N_2-fixing amelioration [4-6]. In Morocco, the inoculation with bacteria strains is little known. It is used only in experimental basis. During the last decades, harvesting chickpea has been gradually extended from semi-arid to arid areas where environmental conditions have negative effects on the establishment of functional and efficient N_2-fixing symbioses [7]. Although non-native rhizobia strains could be used as inoculums in order to relive these problems, but these strains are generally less competitive and may result in less efficient N_2-fixing under these drastic conditions. An efficient symbiosis depends first on both partners, the host legume and rhizobia strain as well as the environmental conditions. In response to drought stress, some chickpea genotypes of early plant stage were more droughts tolerant in comparison to others [9]. This variability could be ameliorated in the presence of tolerant rhizobia strains in the growth stage. This may due to its positive effects on relative water content, photosynthesis rate and nutrient uptake under this condition [5]. Several studies have reported that phosphate solubilizing bacteria (PSB) could enhance crop yields via their high ability to solubilize the applied phosphates as well as those fixed in the soil [10]. Indeed, beside its N_2-fixing, several rhizobia strains have shown important activity of inorganic phosphorus solubilization to the plant [11]. This capacity can be visually assessed by using selection methods based on solid media with different forms of phosphate complex. Generally, micro-organisms solubilizing inorganic P produce organic acids with low molecular weight such as carboxylic and ketogluconic acids [12]. This operation is also responsible for the reduction of the pH of the media [13]. In order to produce highly efficient N_2 fixing chickpea-rhizobia symbioses that could be recommended in the arid and semi-arid regions of Morocco and grow even under stressful conditions, the objective of the present work was to study several characteristics of rhizobia strains nodulating chickpea (*Cicer arietinum* L.) and to select competitive native ones taking into account their tolerance to salinity, drought, temperature, acidity as well as P solubilizing ability.

2. Material and methods

2.1. Strains isolation

The strains isolation has been achieved from the root nodules of chickpea grown in the experimental field of the National Institute of Agronomical Research (INRA), Khemiss Zemamra (Main road Ndeg. 11: El Jadida – Essaouira/Morocco). The plants were harvested their root nodules were detached, washed and then surface-sterilized by immersion in a sodium hypochlorite solution 6% during 1 min and then rinsed several times with sterile distilled water. Individual nodules were after that grounded in physiological water and an aliquot of 20 µL was used in Yeast Extract Mannitol (YEM) plates supplemented with Congo red. Thereafter, the plates were incubated for 48h at 28°C [14]. Several media transplantations were adopted in order to purify rhizobia isolates based on their absorbance to Congo red.

2.2. Evaluation of the salinity tolerance

To evaluate salt tolerance of purified rhizobia strains, the isolates were grown in YEM medium with different concentrations of NaCl (171 mM, 342 mM, 513 mM, 856 mM, 1711 mM) and in the control medium with a

concentration of 1.7 mM of NaCl. Three replicates per isolate were performed. Bacterial growth was *assessed* after 48 h of incubation at 28°C.

2.3. Evaluation of the tolerance to pH levels

To study the effect of pH on bacterial growth, five YEM medium were prepared with different pH: 3.5, 4, 6.8, 9 and 11.

2.4. Evaluation of drought tolerance using polyethylene glycol (PEG_{6000})

Drought tolerance was evaluated in YEM plates with different concentrations of PEG_{6000}, resulting in osmotic stresses (Ψw) of 0; -0.08; - 0.16; -0.23 MPa [15]. The equation linking the different parameters of the water potential to different concentrations of PEG_{6000} is:

$$\mathbf{\Psi_w = -(1{,}18 \times 10^{-2})\, C - (1{,}18 \times 10^{-4})\, C^2 + (2{,}67 \times 10^{-4})\, CT + (8{,}39 \times 10^{-7})\, C^2T}$$

With Ψ_w: water potential (MPa), T: incubation temperature (° C) and C: concentration of PEG_{6000} ($g.L^{-1}$).

2.5. Phosphate solubilization in solid medium with TCP as sole P source

To study the ability of rhizobia isolates to solubilize TCP, an aliquot of 20 µL of the bacterial suspensions (~10^4CFU/mL) was placed at each Petri dish compartments divided to N corresponding to N rhizobia strains. The plates contain 22 mL of the NBRIP agar. Three replicates per strain were performed. After 5 days of incubation at 28°C, the phosphate *solubilization of TCP* has been demonstrated by the appearance of a clear halo around the colonies. The phosphate-solubilizing (PS) activity of the isolates was calculated in terms of phosphorus solubilization index (PSI) by using the formula *PSI=A/B*, where *A* is total of diameter of halo (mm) and *B* is the diameter of colony (mm). Isolates showing *PSI≥2* were considered as phosphate-solubilizing bacteria (PSB) [16].

2.6. Phosphate solubilization in liquid medium

For determining the strains ability to release soluble phosphorus in NBRIP liquid medium from TCP, a preculture of each of the rhizobia isolates was prepared to inoculate 20 mL of NBRIP medium (National Botanical Research Institute Phosphate Growth Medium) [17], the composition of medium was: glucose 10(g/L), $Ca_3(PO_4)$ 2.5, $MgCl_2.6H_2O$ 5, $MgSO_4.H_2O$ 0.25, KCl 0.2 and $(NH_4)_2SO_4$ 0.1, pH 7). 1 mL of the preculture was added to each medium. Autoclaved and not inoculated media, served as controls. The inoculated media and controls were incubated in a shaker incubator in 120 rpm at 28 °C under dark conditions. 2 mL of the suspension was aseptically collected every 24 h for pH determination, bacterial growth and the soluble phosphorus determination in the form of orthophosphate.

2.7. Orthophosphate concentration

Orthophosphate concentration was determined using the colorimetric method [18]. It's based on the formation of a blue antimonyl/phosphomolybdate complex. This complex is reduced with ascorbic acid, giving a blue compounds whose absorbance is measured using a spectrophotometer at 700 nm. This absorbance is directly related to orthophosphate concentration.

2.8. Evaluation of bacterial growth

Bacterial growth was followed using a liquid suspension culture of YEM medium. The results are made at an interval of 3 h with the spectrophotometer at 600 nm.

3. Results and discussion

A total of 68 rhizobia strains were isolated from collected nodules, where only 12 strains were passed the infectivity test with chickpea plants. These isolates appear after 24 to 48 hours on YEM agar medium.

3.1. Evaluation of the salinity tolerance

The increasing of the NaCl concentration has decreased rhizobia growth in all of the studied isolates. About 92% of the isolates were able to grow in concentrations from 0 to 855.6 mM NaCl. Only two strains *MRp6* and *MRp8* have tolerated 1711 mM NaCl concentration and classified as more salt tolerant. However, *MRp4* strain was classified as sensitive to high salinity. In general, bacterial growth zone is limited between 0 and 171.1 mM with complete disappearance beyond 171.1 mM. Similar results were reported by [19] who indicated that rhizobia strains can tolerate NaCl concentrations that exceed 342 mM and the limits of salt tolerance considerably vary from species to another and even between strains of the same species. Squartini *et al. [*20] reported that *R. sullae* nodulating *H. coronarium* tolerated NaCl levels between 290-548 mM NaCl. Moreover, Rehman *et al. [*21] found that *Rhizobium* sp. NBRI2505 *Sesbania* was able to grow under high NaCl concentration of 4791 mM. The ability of rhizobial strains to tolerate high salt concentration is due to the accumulation protective organic osmolytes such as such as proline, betaine and glutamate or carbohydrates to maintain the cell turgor and limit the stress damage [22].

3.1.2. Evaluation of the tolerance to different pH levels

Under pH levels between 6.8 and 9, most of the studied strains show optimal growth. However, only 33% of rhizobia isolates have tolerated pH levels of 3.5 and 4. *MRp6* and *MRp8* tolerated a pH level of 11 and did not tolerated acidic pH levels. Rhizobia strains nodulating chickpeas in Morocco could tolerate a pH levels from 5 to 8 [23]. This tolerance is different from one species to another [24]. Indeed, rhizobia strains nodulating species *Melilotus indicus* could tolerate pH levels between 4.5 and 9 [25]. In this sense, Indrasumunar *et al.* [26] reported that *Bradyrhizobium japonicum* strains could tolerate a pH level of 3.8. The physiological and biochemical mechanisms of adaptation of rhizobia under acidic conditions are numerous. These mechanisms include, among others, the exclusion and expulsion of H^+ protons polyamines accumulation [27], high K^+ content and glutamate in the cytoplasm of stressed cells and the change of the composition of lipopolysaccharide [22]. Isolated rhizobia nodulating pea that can tolerate pH values that exceed 11 [28].

3.1.3. Evaluation of the tolerance to different concentrations of polyethylene glycol (PEG_{60000})

The results showed that *MRp6*, *MRp9*, *MRp11*, *MRp12*, *MRp13* and *MRp14* strains tolerated -0.23 MPa PEG_{6000} (Table.1). The induction of the drought in the medium by the polyethylene glycol is a selective activity for tolerant micro-organisms to water deficit. High concentrations of PEG reduced the available water that is important for the physiological needs of the bacteria. *Rhizobium* sp. NBRI2505 *sesbania* subjected to drought stress, tolerated YEB containing 45% polyethylene glycol 6000 (PEG; w/v) for up to 5 days of incubation at 30°C [21].

Table.1 Drought tolerance of the studied rhizobia isolates

Souches	PEG_{6000} Concentrations (-MPa)

	0	0.08	0.16	0.23
MRp1	+++	+++	-	-
MRp2	+++	+++	+++	++
MRp4	+++	+++	+++	++
MRp5	+++	+++	+++	++
MRp6	+++	+++	++	++
MRp8	+++	+++	+	-
MRp9	+++	+++	++	+
MRp10	+++	+++	++	++
MRp11	+++	+++	+++	+++
MRp12	+++	+++	+++	+++
MRp13	+++	+++	+++	+++
MRp14	+++	+++	+++	+++

3.1.4. Tricalcium phosphate (TCP) solubilization in NBRIP agar

According to the results illustrated in figure 1, about 75% of the studied isolates were able to solubilize TCP from halos that have been appeared around their colonies in NBRIP agar. The phosphorus solubilizing index (PSI) varies from 0 to 4.1 in all of the tested strains with significant variation in their behavior. These results have allowed us to divide the tested strains into two groups according to the *PSI* ≥ 2 ratio. Thus, we can distinguish a group of five strains with PSI that vary from 2.2 to 4.1. *MRp6* strains presented the highest phosphate solubilizing capacity in comparison to the other isolates. A second group presented low P solubilization capacities of and consists of seven strains with diameters of halos varying from 0 to 1.5. *MRp10* and *MRp12* were not able to release soluble phosphorus in the media. *The* utilization of phosphorus solubilizing bacteria as inoculants increases P uptake and enhance plants growth [29,30].

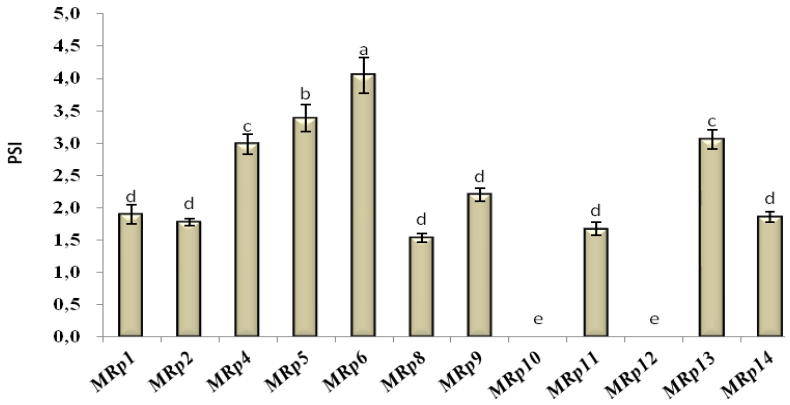

Figure 1. Ability of rhizobia strains isolated to solubilize TCP in NBRIP agar.

3.1.5. Tricalcium phosphate (TCP) solubilization in NBRIP broth

The results showed that all of the studied rhizobia strains grown in NBRIP broth presented strong releases of orthophosphates (Figure 2). We noted a gradual increase of orthophosphate release during the incubation time of each strain. Indeed, *MRp10*, *MRp11*, *MRp12*, *MRp13* and *MRp14* strains released higher concentrations of soluble Pi

compared to the other tested rhizobial strains. The maximum orthophosphate level released by these strains was between 1106 and 1195 mg P L^{-1} after 5 days of incubation. The highest value was recorded for *MRp13* strain. The evolution of the pH during 96 h of incubation allowed us to evaluate the capacity of the tested strains to acidify the medium. The TCP solubilization in the NBRIP broth by the different studied strains has been accompanied by a significant decrease of pH (3.4) from an initial pH 6.8 [31]. The PSB are capable of solubilizing considerable quantities of TCP in the medium by secreting organic acids. P-solubilizing activity of these strains was associated with the release of organic acids and a drop in the pH of the medium.

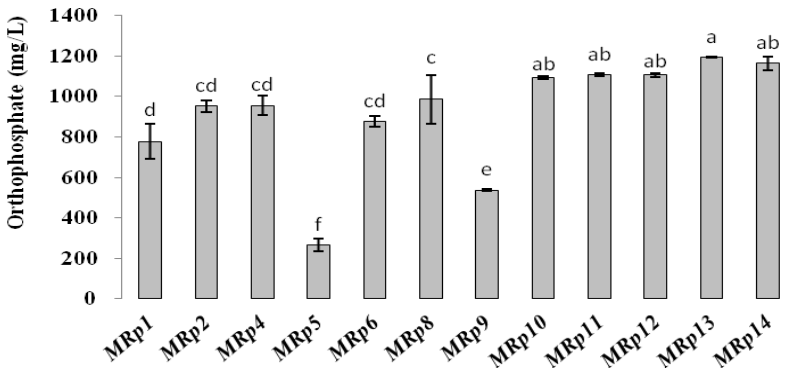

Figure 2. Quantities of released orthophosphate in NBRIP broth by the tested rhizobial strains in the presence of the $Ca_3(PO_4)_2$ as sole P source after 96h of incubation.

The different studied strains showed a different decrease of pH in the medium (Fig.3). *MRp13* has presented a significant pH variation after 24 h of incubation ranging from 6.8 to 4.6. The pH of the medium decreased even more form the initial value after 48 h of incubation to achieve pH values of 3.65. After 96 h of incubation, the pH of the medium has respectively reached the values of 3.42; 3.69; 3.78; 3.82; 3.91; 3.92; 3.93; 3.99; 4.19; 4.24 and 4.41 for *MRp13*, *MRp14*, *MRp10*, *MRPp11*, *MRp12*, *MRp8*, *MRp6*, *MRp4*, *MRp2*, *MRp1*, *MRp5* and *MRp9*. These results were confirmed and explained by the results obtained in the test of the phosphate solubilization and the production of orthophosphate by the studied isolate [32].

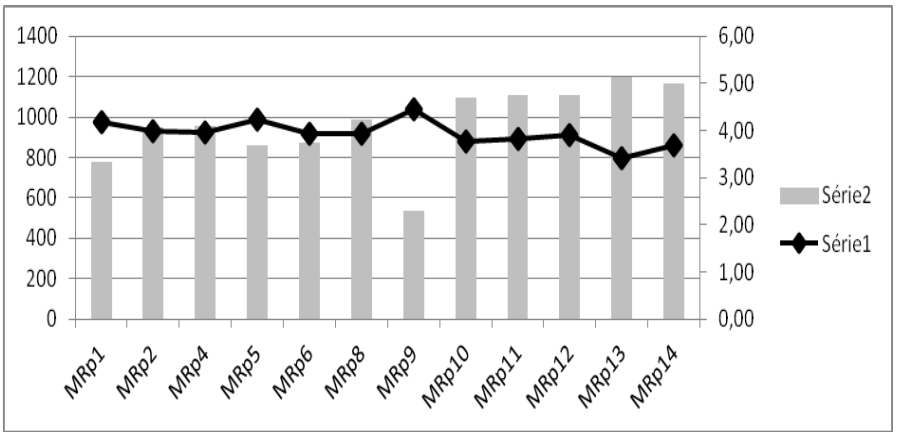

Figure 3. Comparison between the pH in the medium (series1) and the orthophosphate released (series2) after 96h.

3.2. Evaluation of bacterial growth of rhizobia strains in MNBRIP

The results showed that all of the tested rhizobia strains presented different growth speeds in MNBRIP (Fig.4). In general, strains growth was slow during the first 24h, except for the *MRp13* strain which presented a significant

growth. Bacterial growth increases after 48h of incubation for the majority of the studied strains, *MRp9* growth remains very low during 96h of incubation. According to these results, bacterial growth was associated to their ability of the inorganic phosphate solubilization and media acidification [29,31].

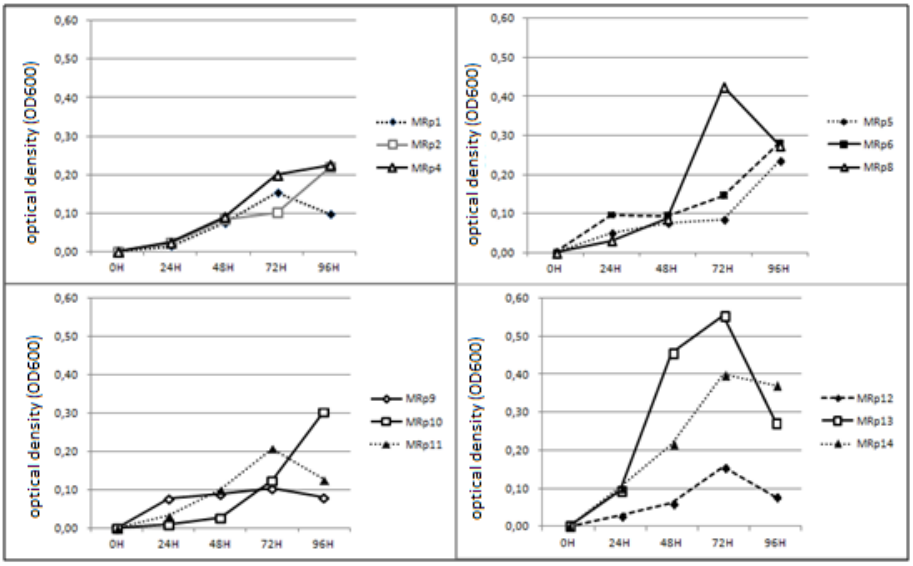

Figure 4. Kinetics growth of the tested rhizobia strains cultured in MNBRIP for 96h.

4. Conclusion

The analysis of the physiological characteristics of the rhizobia isolates has highlighted the physiologic variations existed between all of the tested strains under different abiotic circumstances. The evaluation of major abiotic stress tolerance of selected strains has helped to distinct two rhizobia groups based on their stress tolerance abilities. The most tolerant group is formed by *MRp6* and *MRp8* strains which can tolerate higher NaCl concentrations (1711 mM). As well as their ability to grow in NBRIP agar and broth and the utilization of TCP as sole P source, this solubilization is strongly correlated with the decrease pH of the medium. The results showed that there was high consistence between this solubilizing power and growth speed for all of the studied strains especially those classified as more stress tolerant in comparison with the less tolerant ones.

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