Cultivos Tropicales, Vol. 45, No. 2, April-June 2024, e-ISSN: 1819-4087, p-ISSN: 0258-5936, https://ediciones.inca.edu.cu

Cu-ID: https://cu-id.com/2050/v45n2e05

Original article

Isolation and characterization of rizobia strains from chickpea nodules (*Cicer arietinum* L.)

Aislamiento y caracterización de cepas de rizobios procedentes de cultivares de garbanzo (*Cicer arietinum* L.)

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ABSTRACT : Chickpea (*Cicer arietinum* L.) is a leguminous of wide acceptance in Cuba; it constitutes tolerant specie to adverse environmental conditions, besides their nutritional contribution. Rizobia, which habit inside the chickpea nodules, contribute with benefits to the plant that is why they could be used to elevate their yields and to extend the crop in the country. The objective of this study was to obtain isolates of possible rizobia associated with different chickpea varieties and soil types in Cuba, as well as to characterize them in terms of their tolerance to different pH and temperature values. From seven chickpea varieties sowed in four soil types, 63 bacterial isolates were purified. From them, 11 isolates as possible rizobios by their morphophysiologic and biochemical characteristics were selected. Three of these isolated stood out by tolerate acids and basic pH values and high temperatures, so they could be promissory to constitute active ingredients of new inoculants for this vegetable specie.

Key words: leguminous, growth, selection.

RESUMEN: El garbanzo (*Cicer arietinum* L.) es una leguminosa de amplia aceptación en Cuba, que además de su aporte nutricional tiene como atractivo el ser una especie tolerante a condiciones ambientales adversas. Los rizobios que habitan en el interior de los nódulos de las plantas de garbanzo aportan beneficios al cultivo, por lo que podrían ser utilizados para elevar sus rendimientos y así extender el cultivo en el país. El presente estudio tuvo como objetivos obtener aislados de posibles rizobios asociados a diferentes variedades de garbanzo y tipos de suelos en Cuba, así como caracterizarlos en cuanto a su tolerancia a distintos valores de pH y temperatura. Se purificaron 63 aislados bacterianos obtenidos de siete variedades de garbanzo sembradas en cuatro tipos de suelos. De ellos, se seleccionaron 11 como posibles rizobios por sus características morfo-fisiológicas y bioquímicas. Tres de estos aislados se destacaron por tolerar tanto valores ácidos como básicos de pH y altas temperaturas, por lo que se consideran promisorios para constituir principios activos de nuevos inoculantes para esta especie vegetal.

Palabras clave: leguminosas, cultivo, selección.

INTRODUCTION

Among legumes, chickpea (*Cicer arietinum* L.) stands out as a species of interest for human and animal consumption (1). Studies on the diversity of Plant Growth Promoting Bacteria (PGPB) associated with chickpea have long been limited to the genus *Mesorhizobium*, with the species *Mesorhizobium ciceri*; *Mesorhizobium mediterraneum*; *Mesorhizobium amorphae*, *Mesorhizobium tianshanse*; *Mesorhizobium muleiense* and *Mesorhizobium wenxiniae*.

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Received: 02/08/2022

Accepted: 08/10/2022

Conflict of interest: The authors declare that they have no conflict of interest.

Author contributions: Conceptualization- Marisel Ortega-García. Research- Marisel Ortega-García, Yoania Ríos-Rocafull, Bernardo Dibut Álvarez, María Caridad Nápoles García. **Methodology-** Marisel Ortega-García, Yoania Ríos-Rocafull. **Supervision-** Bernardo Dibut Álvarez, María Caridad Nápoles García. **Initial draft writing, final writing and editing, and data curation-** Marisel Ortega-García.

Subsequently, the presence of new genera and species has been described, which denotes the existence of a wide genetic diversity associated with this crop (2).

As part of the strategy to increase chickpea yields in Cuba, in view of the modification of soil pH and high temperatures caused by climate change (3), the microorganisms naturally associated to this vegetable species could be used as a complement in its nutrition and adaptation, which justifies their isolation and characterization.

The present study aimed to obtain isolates of possible rhizobia associated to different varieties of chickpea and types of soils in Cuba, as well as to characterize them in terms of their tolerance to different pH and temperature values.

MATERIALS AND METHODS

Isolation of isolates

The isolation of possible rhizobia was carried out in 43 sites, from nodules coming from five healthy and vigorous plants of seven chickpea cultivars (Nacional-6, Nacional-24, Nacional-27, Nacional-29, Nacional-30, PJ-94 and Jamu-96), at the flowering stage. The plants were distributed in different productive scenarios in nine provinces of the country (Pinar del Rio, Havana, Artemisa, Mayabeque, Santi Spíritus, Cienfuegos, Granma, Las Tunas and Guantánamo) and on four types of soils (Alitic, Ferrallitic, Fersialitic and Sialitic).

Once the nodules were removed from the plants, they were disinfected in a 5 % sodium hypochlorite solution and rinsed twice with sterile distilled water. Subsequently, they were placed in test tubes containing 15 mL of sterile distilled water and macerated. Each extract was inoculated into the culture medium Yeast Mannitol Agar (YMA) with Congo Red (4) and the plates were incubated at 28-30 °C temperature, for 72 hours. The purification of the microorganisms was carried out in this same culture medium.

Morpho-physiological and biochemical characterization of the isolates

Colony morphology, size and borders were determined by observation under a Leica KL 300 LED stereo microscope (3X), while microscopic and staining characterization was performed by Gram staining (5) and observation under an optical microscope (Leica DM300 (1,000 X magnification)).

The production of acids or bases by the isolates and the production of ketolactase were also evaluated. In the first case, YMA culture medium (4) with bromothymol blue was used and incubation was carried out for seven days at 28-30 °C temperature. For the second, the culture medium Yeast Lactose Agar (YLA) (6) was used.

For the physiological-biochemical characterization, different tests were performed, (7). In all cases, the starting point was inoculation with a 24-hour culture in YMA medium with Congo Red (4). It was incubated for two to five days at a temperature of 28-30 °C and tests were performed in triplicate. Tests included presence of cytochrome oxidase and catalase enzymes, hydrolysis of starch, gelatin and casein, utilization of citrate as a carbon source, indole production from tryptophan, glucose fermentation, sulfur utilization and motility, the latter by the hanging drop method. In addition, the ability of the isolates to degrade cellulose was determined using a culture medium supplemented with 10 g of crystalline cellulose, in which the microorganisms were inoculated. The test was considered positive when a translucent zone was present around the bacterial growth.

Evaluation of the tolerance of the isolates to different pH and temperature values

The LM liquid culture medium (4) was adjusted to different pH values (3; 3.5; 4; 4.5; 5; 5.5; 6; 6.5; 7; 7.5; 8; 8.5; 9 and 9.5) with HCl or NaOH 1N. In all cases, the isolates were inoculated independently and placed on an orbital shaker at 200 rpm for 48 hours at a temperature of 28-30 °C. Visible growth due to turbidity in the culture medium was taken as a positive criterion and absence of growth due to transparency of the medium as a negative criterion. To determine tolerance to different temperatures, YMA culture medium was used, where the isolates were seeded through a central streak and subsequently incubated at 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 and 40 ºC temperature. The test was considered positive when the microorganism grew and negative in the absence of growth. In both determinations, the isolates were inoculated from a 24-hour young culture in YMA culture medium with Congo Red and incubated for two to five days at a temperature of 28-30 °C. The assays were performed in triplicate.

RESULTS AND DISCUSSION

Once the nodules from plants of the different varieties of chickpea were processed, the 63 bacterial isolates that did not fix the Congo Red present in the culture medium, initial indicator of selection for being one of the distinctive characteristics of the rhizobia (5), were purified. The 49.2 % of the samples came from the variety Nacional-29, 22.2 % corresponded to the variety JP-94, while only 3.2 % of the nodules were obtained from the variety N5-HA. Regarding soils, most nodules were collected from plants grown in Ferrallitic soils, with 55.6 % of the samples; 20.6 % were obtained from Alitic soils and 17.5 % from Fersialitic soils, while the least amount was obtained from Sialitic soils (6.3 %) (Figure 1).

One of the fundamental causes that conditioned the isolation of a greater number of microorganisms of the Nacional-29 variety and Ferrallitic soils, is that both were the most representative in the sampling sites. The Nacional-29 variety has stood out for its adaptability to different soils and edaphoclimatic conditions (8), which is why it is the most widespread in Cuba, while the Ferrallitic grouping, more specifically the red Ferrallitic type, occupies

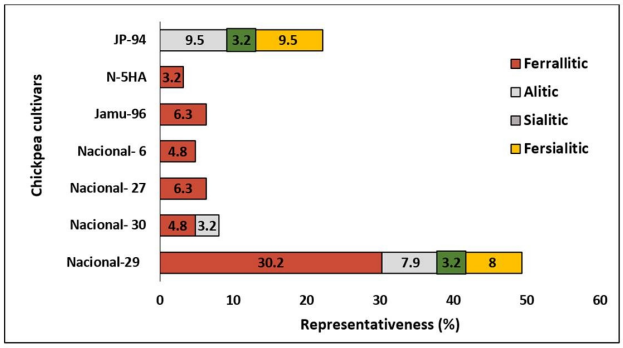

Figure 1. Soil and cultivar representativeness in the isolation of potential rhizobia associated with chickpea

about 700 000 ha and has traditionally been the most used for food production due to its high productivity and the potential it represents for carbon sequestration (9).

Morpho-physiological and biochemical characterization of isolates

The morphology of the isolates purified from the nodules of the different chickpea varieties was heterogeneous and three types of cell morphology could be identified, with a predominance of the coccobacillary form. The most representative group, with 24 % of isolates, corresponded to Gram-positive sporulated coccobacilli; while the smallest group (15 % of isolates) belonged to Gram-negative non-sporulated coccobacilli (Table 1), a morphology that corresponds to that of rhizobia (5). These microorganisms were selected for further characterization.

All isolates showed small to medium-sized colonies, with translucent, whitish or slightly pinkish coloration in the center, without Congo Red absorption and with the mucus typical of rhizobia.

From 11 isolates selected, nine were characterized by colonies of 1 mm in diameter and only two colonies of 4 mm formed. All the colonies were circular, convex and mucilaginous, four presented a beige coloration, three were white, two were whitish or slightly pink in the center and two were translucent, aspects that coincide with those described for the rhizobium family (5). Of these, 8 were obtained from the variety Nacional-29, and the other 3 corresponded to the cultivars Nacional-6, Nacional 5HA and JP-94. In terms of growth rate, seven of the isolates grew between 2 and 3 days, while the other four isolates

Table 1. Micromorphological and color characteristics of bacterial isolates from chickpea plant nodules of different cultivars and soils in Cuba

Morphological and color characteristics	Cultivars/Soils	Number isolates
	Nacional-6/ Ferrallitic	
Sporulated cocobacilli, Gram positive		3
Non-sporulated cocobacilli, Gram positive		1
	Nacional-27/ Fersialitic	
Sporulated cocobacilli, Gram positive		2
	Nacional-27/ Ferrallitic	
Non-sporulated cocci, Gram positive		6
	Nacional-29/ Ferrallitic	
Sporulated cocobacilli, Gram positive		8
Non-sporulated cocobacilli, Gram positive		5
Thick cocobacilli and non-sporulated bacilli, Gram negatives		3
	Nacional-29/ /Alitic	
Gram-negative cocobacilli, non-sporulated thin or round bacilli		3
Sporulated bacilli and cocobacilli, Gram-positive		8
	Nacional-30 Sialitic	
Non-sporulated cocci, Gram positive		4
Sporulated bacilli, Gram negative		5
	Nacional 5HA/ Ferrallitic	
Non-sporulated cocobacilli, Gram negative		1
Gram-positive, sporulated bacilli		6
	JP-94 /Alitic	
Non-sporulated cocobacilli, Gram negative		1
Gram-positive, sporulated bacilli		5
	JP-94 / Ferrallitic	
Non-sporulated cocci, Gram positive		4
	JAMU-96/ Ferrallitic	
Non-sporulated cocci, Gram positive		5
Non-sporulated, Gram-positive, curved bacilli		3

Groups with similar characteristics to rhizobia are highlighted

multiplied between 7 and 9 days. All 11 isolates produced acids in the culture medium and showed a negative result for ketolactase production.

In recent years, it has been shown that populations of bacteria from the rhizobial zone, which do not have the ability to form these structures, coexist with rhizobia in legume nodules (10). In the case of chickpea, although most studies relate the genus *Mesorhizobium* as the most representative, the presence of new genera linked to this crop has been described, such as *Achromobacter xylosoxidans*, *Bacillus subtilis* and *Bacillus thuringiensis* (11), *Burkholderia andropogonis* and *Ochrobactrum ciceri* (12), among others. These results demonstrate that it is possible in the initial isolates to purify microorganisms that do not necessarily belong to the rhizobia group, as was the case in the present investigation.

The different growth rates shown by the 11 selected isolates allowed categorizing seven with rapid growth (2-3 days), which could be members of the Rhizobiaceae family, where the genera *Ensifer*, *Rhizobium* and *Shinella* are included; while the remaining four are included within the slow growth group (4- 7days), where the genus *Bradyrhizobium* is included (10). The production of acid in the culture medium and the negative response to the ketolactose test ratify for these 11 microorganisms their categorization within the Rhizobiaceae family (5).

The 11 isolates selected as rhizobia presented a positive response to the oxidase, catalase, casein hydrolysis, citrate utilization and motility tests; and negative to starch and gelatin hydrolysis, indole production from tryptophan, sulfur utilization from growth in Kliger medium and cellulose degradation. However, they showed differences in sugar fermentation as determined by Methyl Red and Vogues Proskauer tests, as seven of them (R1, R2, R3, R8, R19, R27 and R29) fermented glucose, while four (R1N, R9, R13 and R17) did not (Table 2).

Rhizobia are described in the Manual of Systematics for Bacteria (13) as bacteria with a positive response to the catalase and oxidase test, that do not hydrolyze starch or gelatin but casein, use citrate as a carbon source and have a negative response in the Kliger medium (14) and to the production of indole from tryptophan (15), with strains that use glucose as a carbon source. These characteristics are similar to those exhibited by the eleven selected isolates. Therefore, the physiological characterization performed also reinforces their inclusion within the rhizobial group.

Evaluation of the tolerance of the isolates to different pH and temperature values

None of the 11 isolates grew at pH between 3 and 5, nor at pH 9.5, while the rest of the values did show differences among the microorganisms; meanwhile, at pH values between 6.0 and 7.0 all the isolates grew, while at pH 5.5 only isolates R1, R2, R3 and R19 grew. When the culture medium had a pH of 7.5, most of the microorganisms showed positive growth, with the exception of isolate R8; but higher values (pH between 8.5 and 9.0) affected growth, although it should be noted that R1, R2 and R3 showed greater tolerance and, particularly, isolate R3 was the only one that grew when the culture medium had a pH of 9.0 (Table 3).

Most rhizobia grow at pH values close to neutral (16, 17). However, it has been shown that some strains of this family can tolerate ranges from 3.5 to 9 (18).

Isolates R1, R2 and R3 were able to grow at pH 5.5 and also at values of 8.5; which suggests the presence of adaptation mechanisms that allow it to survive in these conditions (19), an aspect that constitutes an advantage for the practical use of this microorganism as the active principle of a biofertilizer to benefit the chickpea crop, since, if its growth stimulating effect is maintained in the different acidity conditions, it could be used in different types of soil and in different agroecosystems. A possible explanation for the tolerance to acidity or alkalinity is based on the ability of the microsymbiont to maintain internal pH close to neutrality, which could be related to proton exclusion,

Table 2. Physiological-biochemical characteristics of potential rhizobia isolated from chickpea (*Cicer arietinum* L.) plant nodules

Isolates	Ox	Cat	Alm	Gel	Cas	Cit	Ind	VP	RM	Kli	Cel	Mot
R1	+	+	-	-	+	+	-	+	+	-	-	+
R2	+	+	-	-	+	+	-	+	+	-	-	+
R3	+	+	-	-	+	+	-	+	+	-	-	+
R1N	+	+	-	-	+	+	-	-	-	-	-	+
R8	+	+	-	-	+	+	-	+	+	-	-	+
R9	+	+	-	-	+	+	-	-	-	-	-	+
R13	+	+	-	-	+	+	-	-	-	-	-	+
R17	+	+	-	-	+	+	-	-	-	-	-	+
R19	+	+	-	-	+	+	-	+	+	-	-	+
R27	+	+	-	-	+	+	-	+	+	-	-	+
R29	+	+	-	-	+	+	-	+	+	-	-	+

Ox: enzyme oxidase activity, Cat: catalase, Alm: starch hydrolysis, Gel: gelatin hydrolysis, Cas: casein hydrolysis, Cit: utilization of citrate as carbon source, Ind: indole production from tryptophan, VP: glucose fermentation by Vogues Proskauer, RM: glucose fermentation by Methyl Red, Klig: use of sulfur from growth in Kliger medium, Cel: cellulose degradation and Mot: motility

Table 3. Growth of potential rhizobia isolated from chickpea plants at different pH levels

Isolates	pH Values													
	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5	9	9.5
R1	-	-	-	-	-	+	+	+	+	+	+	+	-	-
R2	-	-	-	-	-	+	+	+	+	+	+	+	-	-
R3	-	-	-	-	-	+	+	+	+	+	+	+	+	-
R1N	-	-	-	-	-	-	+	+	+	+	-	-	-	-
R8	-	-	-	-	-	-	+	+	+	-	-	-	-	-
R9	-	-	-	-	-	-	+	+	+	+	+	-	-	-
R13	-	-	-	-	-	-	+	+	+	+	+	-	-	-
R17	-	-	-	-	-	-	+	+	+	+	+	-	-	-
R19	-	-	-	-	-	+	+	+	+	+	+	-	-	-
R27	-	-	-	-	-	-	+	+	+	+	-	-	-	-
R29	-	-	-	-	-	-	+	+	+	+	-	-	-	-

Growth (+) no growth (-)

Tabla 4. Growth of potential rhizobia, isolated from chickpea plants, at different temperature values

Isolates	Temperature (°C)															
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
R1	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-
R2	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-
R3	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-
R1N	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-
R8	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-
R9	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-
R13	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-
R17	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-
R19	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-
R27	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-
R29	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-

Growth (+) no growth (-)

increased cytoplasmic buffer capacity, or maintenance of high potassium and glutamate concentrations (20).

None of the 11 isolates grew at 25 and 27 ºC, nor did they grow at 40 ºC. However, from 30 ºC to 35 ºC, all the microorganisms developed well and between 28 and 36 ºC between 63 and 82 % of the isolates grew, which could be related to their origin from plants cultivated in the edaphoclimatic conditions of Cuba, where the temperature oscillates between 28 and 35 ºC. Isolates R1, R2 and R3 stood out for their growth at high temperatures, since they tolerated ranges from 29 to 38 ºC and, particularly R3, the only isolate that grew at 39 ºC (Table 4).

According to several authors, rhizobia can grow at temperatures between 28-30 °C (19). Other investigations have yielded results similar to those achieved in the present study, and even higher, where isolates from nodules of *Vigna unguiculata* tolerated up to 45 ºC (18), results that suggest an adaptation to the high temperatures characteristic of the areas of origin.

The tolerance of some strains to different pH and temperatures can favor their multiplication in the rhizosphere and contribute to a greater colonization of the species, and with this, to its success as a plant growth promoting bacterium. In this regard, authors such as (21, 22) pointed out that these factors of survival, persistence and competitiveness give rhizobia greater possibilities of surviving and competing with other soil bacteria, which enhances the colonization of legume roots and the fixation of atmospheric nitrogen.

CONCLUSIONS

- Sixty-three bacterial isolates were obtained from seven chickpea varieties planted in four types of soils. From these, 11 were selected as potential rhizobia because of their morpho-physiological and biochemical characteristics.
- Isolates R1, R2 and R3 showed morphological, color and physiological characteristics common to those described for rhizobia, as well as greater tolerance to the pH and temperature conditions studied, making them promising microorganisms as active principles of new inoculants to benefit the chickpea crop.

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