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**ORIGINAL ARTICLE**

# ISOLATION AND DIAGNOSIS OF *RHIZOBIUM* OF COWPEA PLANT AND THEIR EFFICIENCY IN HOST PLANT INFECTION AND NITROGEN FIXATION

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**Abstract:** The research is aimed to isolate *Rhizobium* bacteria of cowpea plants from different environmental areas, to study their microbial and cultivation characteristics, and to measure their efficiency in fixing atmospheric nitrogen and infecting the host plant. Ten local isolates of *Rhizobium* were obtained from cowpeas cultivated in Basra and Maysan. The results showed difference in size, color of root nodules of cowpea plant for samples taken from different regions and their efficiency in nitrogen fixation and infection of host plant. The efficient isolates were taken from maysan soil. The $R_6$ isolate isolated from Al-Qalaa area (Maysan) was superior in fixing atmospheric nitrogen as it reached 18.42 mg *N*. 100 $ml^{-1}$ of liquid medium and forming active nodules on cowpea plant at a rate of 6.67 nodule. $Pot^{-1}$.

***Key words*:** *Rhizobium*, Isolation, Diagnosis, Cowpea .

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## 1. Introduction

Nitrogen content in soils were a limited factor for production of crops in many developing countries. As the nitrogen fixation by *Rhizobium* bacteria plays an important role in providing an adequate amount of nitrogen to the legumes. *Rhizobium* is characterized by its specialization in infecting leguminous plants, which is a prerequisite for developing inoculants to increase crop production, including cowpea. Symbiotic relationship between rhizobia and legumes is a major source of stabilized nitrogen (ammonia) in the biosphere, and this process increases agricultural productivity which reduces dependence on nitrogenous fertilizers [Patel and Minocheherhomji (2018), Slomy *et al.* (2019)] and reduces pollution risks caused by use of chemical fertilizers in large quantities and production costs [AL-Taey and AL-Musawi (2019)]. Adams *et al*. (2018) Bongo and Pietr (2019) showed the importance of studying *Rhizobium* bacteria and their infection to cowpea plants under arid and semi-arid conditions. It was mentioned that there are strains of *Rhizobium* bacteria that can infect cowpea plant and form root nodules under the conditions of the Sub-Saharan Africa (SSA), including nearly 4000 bacterial strains that have been diagnosed as belonging to *Brady Rhizobium elkanii*, *B. japonicum* and *B. yuamingense* isolated from soils and roots of cowpea plants grown in multiple regions of SSA. Several of them can be used as efficient inoculant in inducing infestation and nitrogen fixation on the hostplant. The strains of *Rhizobium* were capable to produce nodules when induced *Rhizobium* as inoculants and resulted in significant changes in cowpea plant characteristics. Growth of the legumes was determined in plants inoculated with *Rhizobium* sp. SOY7 was statistically significant ($p < 0.05$) in Total Nitrogen, Phosphorous, Sulfur , Zinc (0.10 % , 25.9 ppm , 41.1ppm , 5.20 ppm) respectively compared with non-inoculated (controls)

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[Nushair *et al*. (2017)]. The study aims to isolate specialized local *Rhizobium* bacteria on the cowpea plant from different environmental areas and to study their ability to fix nitrogen and infect the host plant.

## 2. Materials and Methods

Cowpea plant was cultivated in an agricultural field in Abo Al-Khasib (Basra Governorate ) in clay soil with high salinity (16.14 ds.m$^{-1}$) as field was planted 3 farrow each one 5 meters long and 30 cm wide, and after the plant reaches the flowering stage. Growing plants were rooted in suitable soil moisture. Three pink nodules were obtained coded $R_1$, $R_2$, $R_3$. Also, seven large pink root nodules were obtained for cowpea planted in different areas of Maysan Governorate, coded $R_4$, $R_5$, $R_6$, $R_7$, $R_8$ from alcalaa city and $R_9$, $R_{10}$ from alkhder city.

### 2.1 Isolation of *Rhizobium* bacteria

Ten pink nodules of cowpea plant were used and sterilized with 95% ethyl alcohol and 5% Clorox, after which they were washed with sterile distilled water seven times to remove the sterilization residue, according to Beck *et al*. (1993). Sterilized nodules were transferred by sterile forceps to sterile test tubes with two drops of sterile distilled water, then nodules were crushed at the bottom of the tube by end of a sterile glass rod until the slurry was formed . A small amount of slurry was taken with a sterile inoculation loop and streaked on surface of culture medium of Yeast Extract Mannitol Agar (YEMA) containing Congo Red stain and the plates were incubated inverted at 30°C for 3-7 days. The emergence of mucous white colonies on surface of culture media is an indication of the presence of *Rhizobium* colonies. *Rhizobium* colonies were purified by re-cultivation on the same medium (YEMA), then colonies were planned on slant nutrient medium and were preserved at 4°C for subsequent studies according to Brenner *et al.* (2005) and Beck *et al*. (1993).

### 2.2 The ability of isolates to fixing atmospheric nitrogen

Efficiency of isolates in fixing atmospheric nitrogen was determined by preparing yeast extract broth medium and mannitol (YEMB). It placed in test tubes and inoculated with 1 ml of *Rhizobium* bacteria inoculated with a concentration of $10^8$ cfu.ml$^{-1}$, then media was shaken by hand and placed in the incubator at 30°C for 12 days and then placed in a water bath at a temperature of 60°C, after which medium was digested with the acid mixture (96% concentrated sulfuric acid and 4% perchloric acid) and the total nitrogen was determined by the Kjeldal method [FNCA (2006)].

### 2.3 Infection of host plant

This experiment was carried out with the aim of finding out ability of isolates to infect cowpea (the host plant) and form root nodules. Prepare pure sand by immersing it in 0.01 N hydrochloric acid. Then wash sand with distilled water several times until the chlorine disappears from the washing leachate, sand was dried in an electric oven at a temperature of 100°C and put in pots of 1 kg Potted$^{-1}$, planting with sterilized cowpea seeds, then inoculated with 1 ml of the ten inoculant its concentration ($10^8$cfu.ml$^{-1}$) under sterilization according to Al- Amin (1999) and Beck *et al*. (1993).

The experiment was designed with three replicates, and a control treatment without inoculation watered pots with nutrient solution (Broughton and Dillworths nitrogen-free mineral solution) according to Beck *et al*. (1993). After germination and growth for 30 days, whole plants were extracted from the pots using a light stream of water, roots were washed carefully and examined to notice that root nodules were on them and recorded their number and color for each plant.

## 3. Results and Discussion

The results showed that growth rates of isolated bacteria on the cultural medium YEMA at 28°C during the incubation period were 3-7 days (Table 1). Isolated bacteria were also distinguished through some of their cultivation characteristics that the shapes of their colonies were all viscous, convex, smooth and mucous at 100%. As for the color on the medium, it varied between white and cream number of isolates whose colonies showed a transparent white color were 8, while 2 isolates showed white to creamy color. The microscopic properties of bacterial cells showed that their short bacillus cells, gram negative and motility. The results showed that individual colonies of the isolated bacterial cells were white when grown on yeast extract - mannitol solid YEMA-CR containing Congo red stain (Fig. 1). Since the colonies of most of those isolates did not absorb this dye by 80%, while 20% gave a pinkish-red color, and this means that colonies belong to the genus *Rhizobium*, which is characterized by not absorbing the Congo red dye , and these results are in agreement with Ali *et al* . (2019) and Nushair *et al*

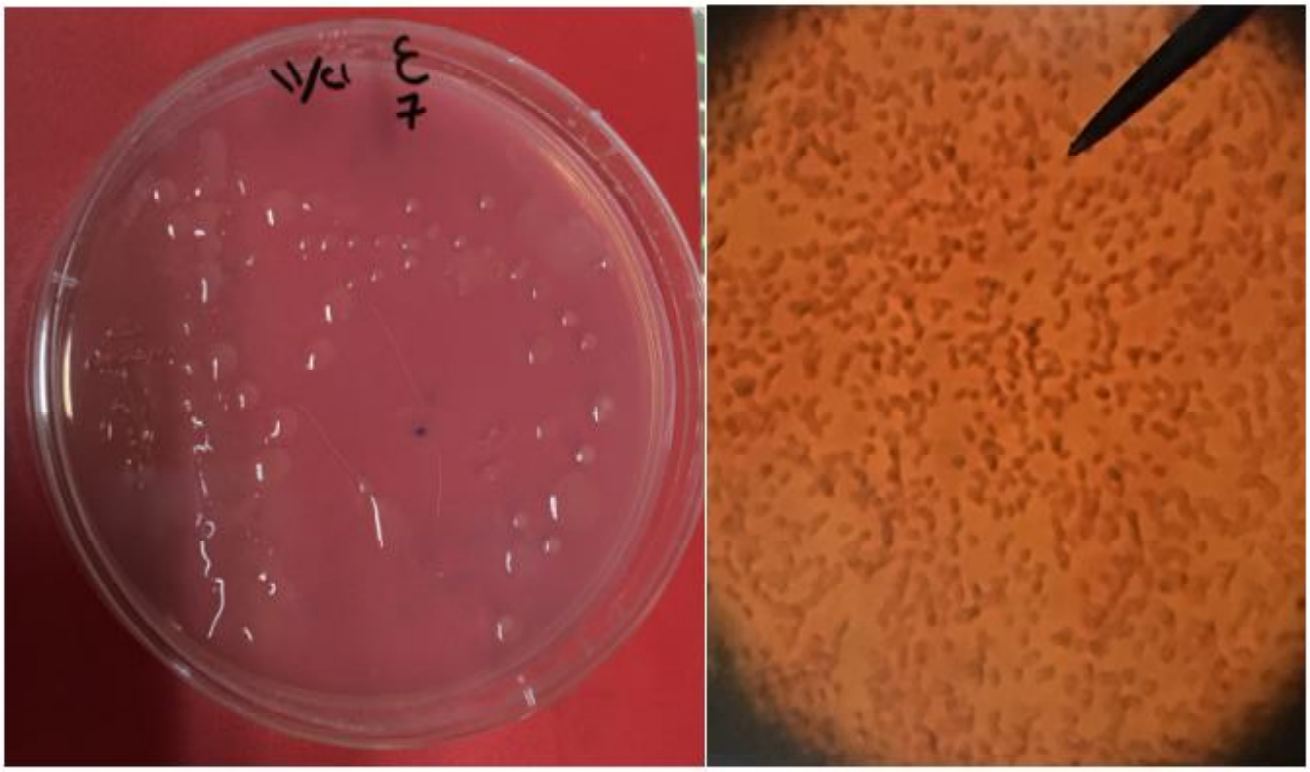

**Fig. 1:** Colony of *Rhizobium* bacteria on YEMA-CR and their shape under microscope

**Table 1:** Biological and cultural properties of bacteria isolated from roots of cowpea plants cultivated in different regions.

| | **Adjective** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Isolates code** | **Cultivated properties** | | | | | | | **Microscopic properties** | | |
| | **growth convex** | **Colony** | **Texture** | **Mucus** | **Congo red stain** | **Bromothymol blue stain** | **color** | **shape** | **movement** | **Cram stain** |
| $R_1$ | Fast | Convex | Smooth | Mucous | white | yellow | white | Short bacillus | Motility | - |
| $R_2$ | Slow | Convex | Smooth | Mucous | white | yellow | white | Short bacillus | Motility | - |
| $R_3$ | Slow | Convex | Smooth | Mucous | Pink | blue | white | Short bacillus | Motility | - |
| $R_4$ | Slow | Convex | Smooth | Mucous | white | blue | Creamy | Short bacillus | Motility | - |
| $R_5$ | Slow | Convex | Smooth | Mucous | Pink | blue | white | Short bacillus | Motility | - |
| $R_6$ | Slow | Convex | Smooth | Mucous | white | blue | white | Short bacillus | Motility | - |
| $R_7$ | Slow | Convex | Smooth | Mucous | white | blue | white | Short bacillus | Motility | - |
| $R_8$ | Slow | Convex | Smooth | Mucous | white | blue | Creamy | Short bacillus | Motility | - |
| $R_9$ | Slow | Convex | Smooth | Mucous | white | blue | white | Short bacillus | Motility | - |
| $R_{10}$ | Slow | Convex | Smooth | Mucous | white | blue | white | Short bacillus | Motility | - |

**Table 2:** The ability of *Rhizobium* isolates to fixing atmospheric nitrogen (mg. 100 ml $^{-1}$).

| Isolate code | Total nitrogen fixation in the medium (mg . 100 ml $^{-1}$) |
|---|---|
| $R_1$ | 5.01 |
| $R_2$ | 3.37 |
| $R_3$ | 6.12 |
| $R_4$ | 11.35 |
| $R_5$ | 9.78 |
| $R_6$ | 18.42 |
| $R_7$ | 3.19 |
| $R_8$ | 15.31 |
| $R_9$ | 10.03 |
| $R_{10}$ | 3.11 |

**Table 3:** Number of root nodules formed on cowpea plant inoculated with different *Rhizobium* isolates.

| Isolates code | Rate of total number of nodules | Rate of effective nodules |
|---|---|---|
| $R_1$ | 1.33 | .33 |
| $R_2$ | .0000 | .0000 |
| $R_3$ | 18.67 | 6.33 |
| $R_4$ | 2.67 | 1.33 |
| $R_5$ | .0000 | .0000 |
| $R_6$ | 15.33 | 6.67 |
| $R_7$ | .0000 | .0000 |
| $R_8$ | 12.33 | 5.00 |
| $R_9$ | 11.67 | 5.67 |
| $R_{10}$ | .0000 | .0000 |
| $R_0$ control | .0000 | .0000 |

(2017). They indicated that colonies of *Rhizobium* bacteria do not absorb red color of Congo red dye. Results of Table 1 showed that bacterial isolates growing on the YEMA medium to which the bromothymol blue dye was added, showed a clear change in the color of media after the end of incubation period by changing the green to yellow or blue which distinguishes fast-growing groups, especially the genus *Rhizobium*, from slow-growing such as the genus *Brady Rhizobium*, as well as rapid growth of bacteria through their appearance in the growth medium, eight bacterial isolates changed color of culture medium from green to blue, while 2 isolates changed color of medium to yellow, and these results are consistent with findings of Sharma *et al*. (2011) and Kapembwa *et al*. (2016). It may be attributed to ability of some isolates to produce compounds that make medium acidic, so it becomes yellow in color, or other compounds that make it alkaline and turns blue.

The results showed that all isolates are able to fix nitrogen in varying quantities (Table 2). $R_6$ isolate gave highest fixation rate of 18.42 mg.100 ml $^{-1}$ while isolate $R_{10}$ gave lowest fixation rate of 3.11 mg.100 ml $^{-1}$. The difference of isolates in their ability to fix nitrogen may be attributed to the different environment from which they were collected, which affects the properties and function of that bacterium, especially with regard to the gene expression of this trait [Kapembwa *et al*. (2016) Nushair *et al*. (2017 )].

Table 3 shows effect of inoculation with different *Rhizobium* isolates on number of root nodules formed on cowpea plant 30 days after germination. It is noticed that highest rate of effective root nodules (pink) on plant was due to the effect of isolation ($R_6$) isolated from Al-Qalaa area (Maysan Governorate), reaching (6.67) nodule. Plant$^{-1}$. While lowest effective root nodules rate was (0) due to effect of isolates $R_0$, $R_2$, $R_5$, $R_7$, $R_{10}$ .

## 4. Conclusions

This data showed that a less number of local *Rhizobium* strains were effective in infecting host plants due to environmental conditions such as salinity, lack of vegetation cover, drought and pollutants spread in Iraqi soils. Thus confirms to fact that were not possible to completely rely on the native Rhizobia to infect the root of leguminous plants and nodules formation.

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