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MORPHOLOGICAL, BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF 25 *RHIZOBIUM LEGUMINOSARUM* ISOLATES FROM FABA BEAN IN MENOFIA GOVERNORATE, EGYPT

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ABSTRACT: We collected root nodules of faba bean plants from nine different area in Menofia Governorate. Twenty five isolates were collected from faba bean as a host trap. We identified our tested isolates for morphological characteristics (motility, carbol fuchsin stain and gram stain) and biochemical characteristics (acid or alkli production, utilization of different carbon sources, pH and NaCl tolerance, and starch and urea hydrolysis). We tested them on molecular level by amplification of 16s rRNA gene. All tested isolates came to be motile, gram-negative, not able to absorb Congo red. Results of biochemical testes showed that all tested isolates were acid producers, fast growers, able to survive on NaCl concentration ranging from 0.5 to 5%. All of them were able to grow in presence of wide range of pH (5 to 8). 16s rRNA gene came to be same size (about 1500 bp) in all Rhizobial isolates.

Key words: *Rhizobium leguminosarum*, morphological, biochemical, faba bean, 16s rRNA.

INTRODUCTION

Rhizobia encompass a range of bacterial genera, including *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium*, which are able to establish a symbiosis with leguminous plants. They elicite the formation of specialized organs, called nodules, on roots of their hosts, in which they reduce atmospheric nitrogen and make it available to the plant. Symbiotic nitrogen fixation is an important source of nitrogen, and the various legume crops and pasture species often fix as much as 200 to 300 kg nitrogen per hectare (Peoples *et al.*, 1995). So far, 21 bacterial genera have been identified as nodule-forming microsymbionts (Wang *et al.*, 2019a). These microsymbionts are distributed among major bacterial genera. *Vicia faba* L. (*V.f)* or faba bean is a grain legume of great importance in world agriculture. Its ability to fix nitrogen through symbiosis with *Rhizobium leguminosarum* in its root nodules, but most crucially for its role as a staple dietary protein source in North African and Middle Eastern cultures. Faba bean (*Vicia faba L.*, broad bean, horse bean) is grown worldwide in cropping systems as a grain and green-manuren legume. The faba bean contributes to the sustainability of cropping systems via: (1) its ability to contribute nitrogen (N) to the system via biological N2 fixation, (2) diversification of systems leading to decreased disease, pest and weed build-up and potentially increased biodiversity, (3) reduced fossil energy consumption in plant production, and (4) providing food and feed rich in protein (FAOSTAT, 2008). The beneficial association between rhizobia and legumes has been known for more than a century. Nitrogen is an essential component of all amino acids and nucleic acids, thus making it an important plant nutrient element. Although the atmosphere consists of 78.1% N2 gas, plants cannot use it unless it is converted into a usable form (Ferguson *et al.*, 2010). Biological N2 fixation (BNF) is a free source of N that can be exploited by resource-poor farmers for increased crop yields (Giller and Cadisch, 1995), making it one of the most important micro-biological processes on earth; globally, 33– 46 Tg of N $year^{-1}$ is contributed by the legume-rhizobia symbiosis (Herridge, 2008).

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We conducted this research to study morphological, biochemical and molecular characteristics of 25 *Rhizobium leguminosarum* isolates that collected from Faba bean in nine different locations of menofia governorate.

MATERIAL AND METHODS

This study is carried out on 25 Rhizobium isolates that were isolated from the roots of faba bean plants, from nine locations in Menofia Governorate (Shebin Elkom –Tala - Berkit Elsaba –Ashmoun – Menouf – Elshohada – Quisna – Elbagour and Elsadat City).

• Collection of root nodules

Root nodules were collected from young and healthy plants (*Viciae faba L.*) according to Deshwal and Chaubey (2014).

• Isolation of Rhizobia from nodules on selective media (YEM)

Healthy nodules were detached from the faba bean roots and further rhizobial bacteria were isolated (Vincent, 1970). The detached root nodules were washed in tap water to remove the adhering soil particles from nodule surface. Nodules were dipped in 0.1% mercuric chloride (HgCl2) solution for 30 sec and later washed successively ten times with sterilized distilled water to remove the traces of toxic HgCl2. Sterilized nodules were transferred to test tube containing 5 mL sterilized distilled water. These nodules were crushed with the help of sterilized glass rod to obtain a milky suspension of bacteria. These were streaked asepticaly on yeast extract mannitol agar (YEMA) plates. The plates were incubated at 28°C for 24 hours. Rhizobium colonies appeared white, translucent, elevated and mucilaginous. The single colony isolates were picked up and transferred to yeast extract mannitol agar (YEMA) slants and kept in refrigerator to be used later for further characterization according to Deshwal and Chaubey (2014).

Morphological identification

• Motility test

It was conducted on plates containing the following media in Table (1) according to Crabtree and Hinsdill (1974) .

1-Using aseptic techniques we picked up a small amount of Rhizobial cultures using inoculating needle and stab the motility agar down in the center of the plate.

2-Incubate the plates at 28°c for 48 hrs.

3-Examine all plates to see whether the culture has grown only in inoculation position (non-motile) or has spread throughout the soft agar (motile).

• Carbol fuchsine test

Prepare the carbol fuchsine stain by mixing the following 2 solutions according to Crabtree and Hinsdill (1974).

Solution A: 0.2 g fuchsine and 10 ml 95% ethanol.

Solution B: 5 g phenol and 90 ml distilled water.

Procedure

1. Prepare slides by applying sample in a thin smear and heat fixing the sample on the slide.
2. Flood the entire slide with Carbol Fuchsin stain.
3. Heat the slide using a Bunsen burner until it is steaming.
4. Maintain steaming for 2 minutes by using heat.
5. Allow the slide to cool briefly and rinse the slide with water and shake off any excess moisture.
6. Rinse thoroughly with water and allow to air dry.
7. Examine the smear microscopically using a 100X immersion oil.

• Gram stain test (Gram 1884)

The stain involves 3 major steps/processes that include

1. Staining with crystal violet (a water-soluble dye).
2. De-colorization (using ethanol/acetone).
3. Counterstaining (using Safranin).

Smear preparation

It is important to note the thickness of the sample smear on the slide. The smear should not be thick or too thin.

Table (1): Motiliy Media g/L.

Glucose	2.0 g
Peptone	3.0 g
Yeast extract	2.0 g
Agar	5.0 g
Distilled water	1000 ml

Lable the slide

Bacterial smear was prepared on the slide by introducing a drop of saline on the sample and then mixing. This should then be left to air dry before heat fixing by carefully passing the slide through the binsen burner (avoid burning the sample).

Staining procedure

1. Flood the slide with crystal violet staining reagent for about 1 minute.
2. Wash the slide using a gentle, indirect stream of tap water for about 2 seconds, flood the slide with a mordant (Gram's iodine) then wait for a minute.
3. Wash the slide again in a gentle, indirect stream of tap water for about 2 seconds.
4. Flood the slide with the ethanol then wait for 15 seconds. This can also be done by adding a drop by drop to the slide until the ethanol running clear from the slides.
5. Flood the slide using safranin counterstain and wait for about 30 seconds.
6. Wash the slide using a gentle and indirect stream of tap water to a point where the color appears in the effluent and then blot dry by absorbent paper.
7. Add a drop of immersion oil on the stained sample and examine under the light microscope at (100 X 10) x.

Biochemical identification

• Acids or alkali production

The production of acids or alkali were evaluated on YEMA containing 25 µg ml^{-1} bromothymol blue (BTB) as reaction indicator Lupwayi and Haque, (1994).

• Utilization of carbon source

All tested isolates were grown on seven different sugars as a sole carbon source (Glucose, Fructose, Sucrose, Lactose, Sorbitol, Mannose, and Maltose). Stock solution of each carbohydrate was prepared at 10% (w/v) in sterile distilled water. 10 ml of the carbohydrate stock solution was added to 90 ml of the carbohydrate free basal medium and isolates growth was examined after 3 days, according to Somasegarian and Hoben, (1994) .

• pH tolerance

Cells were allowed to grow in 10ml of YEMB. The capacity of each rhizobial isolates to grow on acidic and alkaline media was determined by inoculating a loopful of each tested isolate on YEMA adjusted at a pH of 4.0, 5.0, 6, 8.0, and 9.0 using sterile 1N HCl and NaOH before autoclaving and incubated at 30^{o}C for 3 days, according to McVicar *et al.,* (2005).

• Salt tolerance

Each isolate was allowed to grow in 10 ml YEMB loopful of each inoculum was inoculated on plates containing YEMA adjusted to 0, 0.5, 1, 2 and 4% (w/v) concentrations of NaCl and incubated at 30^{o}C for 3 days, according to McVicar *et al.,* (2005).

• Starch hydrolysis test

Starch hydrolysis test was performed to determine the ability of isolates to use starch as a carbon source. This medium was inoculated with Rhizobium and analyzed for starch utilization. Iodine test was used to determine the capability of isolates to use starch. Drops of iodine solution (0.1 N) were spread on 24 hours old cultures grown on Petri plates. Formation of blue color indicated non utilization of starch, according to De Oliverira *et al*, (2007).

- **Urea hydrolysis test**

YEM broth was amended with 2% (w/v) urea and 0.012% phenol red to check the urea hydrolysis. The broth was inoculated with log phase cultures of isolates and incubated at 28°c for 48 hours and observed for the production of color, according to Lindstrom and Lehtomaki, (1988).

Molecular identification

Preparation of Total (Genomic and plasmids) DNA from Rhizobial isolates (Wilson 2001).

Materials

- TE (Tris, EDTA) buffer.
- 10% sodium dodecyl sulfate (SDS).
- 20 mg/ml proteinase K (stored in small single-use aliquots at -20°c).
- 5 M NaCl.
- CTAB/NaCl solution.
- 24:1 chloroform/isoamyl alcohol.
- 25:24:1 phenol/chloroform/isoamyl alcohol.
- Isopropanol.
- 70% ethanol.

1. Inoculate a 5 ml liquid culture with the studied bacterial strain. Grow in conditions appropriate for that strain (i.e., appropriate medium, drug selection, temperature) until the culture is saturated.
2. Spin 1.5 ml of the culture in a microcentrifuge for 2 min. Discard the supernatant.
3. Resuspend pellet in 567 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate at 37°C for 1 hr. The solution should become viscous as the detergent lyses the bacterial cell walls.
4. Add 100 µl of 5 M NaCl and mix thoroughly. This step is very important since a CTAB–nucleic acid precipitate will form if salt concentration drops below about 0.5 M at room temperature (Murray and Thompson, 1980). The aim here is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution.
5. Add 80 µl of CTAB/NaCl solution. Mix thoroughly and incubate at 65°C for 10 min.
6. Add an approximately equal volume 0.8 ml of chloroform/isoamyl alcohol, mix thoroughly, and spin in a microcentrifuge at 10000 rpm for 4 to 5 min.

This extraction removes CTAB–protein/polysaccharide complexes. A white interface should be visible after centrifugation.

7. Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge at 1000 rpm for 5 min. With some bacterial strains the interface formed after chloroform extraction is not compact enough to allow easy removal of the supernatant. In such cases, most of the interface can be fished out with a sterile toothpick before removal of any supernatant. Remaining CTAB precipitate is then removed in the phenol/chloroform extraction.
8. Transfer the supernatant to a fresh tube. Add 0.6 vol isopropanol to precipitate the nucleic acids (there is no need to add salt since the NaCl concentration is already high). Invert the tube back and forth until a stringy white DNA precipitate becomes clearly visible.
9. Wash the DNA with 70% ethanol to remove residual CTAB and respin for 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet in lyophilizer.
10. Redissolve the pellet in 100 µl TE buffer.

Amplification and sequencing of 16S rRNA as universal gene

The DNA of bacterial isolates were amplified with the universal primers fD1 (5'AGAGTTTGATCCTGGCTCAG3') and rP2(5'ACGGCTACCTTGTTACGACTT3') (Tsuzuki *et al*., 2008). Polymerase chain reaction was performed in 50-µL reaction volume containing 100 ng DNA, 25 µL Maxima Hot Start PCR Master Mix and 20 µM of forward and

reverse primers. Amplifications were performed with the following PCR conditions: initial denaturation at 95°C for 10 min, 35 cycles with denaturation 95°C for 30 s, Annealing at 58°C for 1 min, Elongation at 72°C for 1 min 30s for every cycle and 10 min final extension at 72°C.

RESULTS AND DISSCUSION

We collected 25 isolates of *Rhizobium leguminosarum* bacteria from faba bean nodules. Those were collected from different locations in Menoufia Governorate. We tested obtained isolates for morphological and biochemical characterization.

Morphological characteristics

To test motility of isolated *Rhizobium leguminosarum*, we inoculated them on soft agar plates and incubated them at 28°C for 24 and 48 hrs. All tested isolates came to be motile at both tested times as presented in Figure (1). Our data came to be in coherence with many authers such as Fathy *et al*., (2021) on testing their isolates , This consider one of the specific characters of *Rhizobium leguminosarum* bacteria as motile.

- Data presented in the Table (2) clearly showed that all tested Rhizobial isolates were positive to fuchsin stain as presented in Figure (2). This response has been approved and published before by Yasmin *et al*, (2021).
- Moreover, all strains appeared to be negative to gram stain the same Table (2) . These facts have been reported by Makonnen and Debebe, (2021) in their research on *Rhizobium leguminosarum* isolates from faba bean nodules that planted in Ethiopia.

Biochemical characteristics

Data presented in Table (3) clearly showed that all isolates are acid producing and giving rise to yellow color.

Data presented in Table (4) clearly showed the ability of all tested Rhizobial isolates to use and utilized all sugars presented in the above table as sole carbon source in YEMA media. All isolates were able to use them, but mannitol was the best carbon source for their growth. This finding came to be coincided with what Hassan *et al* (2015) published. These results also agreed with what Melak *et al* (2018) found in their research on *Rhizobium leguminosarum*, where they tested their growth on wide ranges of carbon sources (galactose, maltose, sorbitol and arabinose). There was no diversity between tested isolates in utilization of used sugars as carbon source. This finding is in agreement with what Legease and Assefa (2014) published. We have to mention that Rhizobial growth have shown the ability to utilize a wider ranges of carbon sources as presented in the above Table (4).

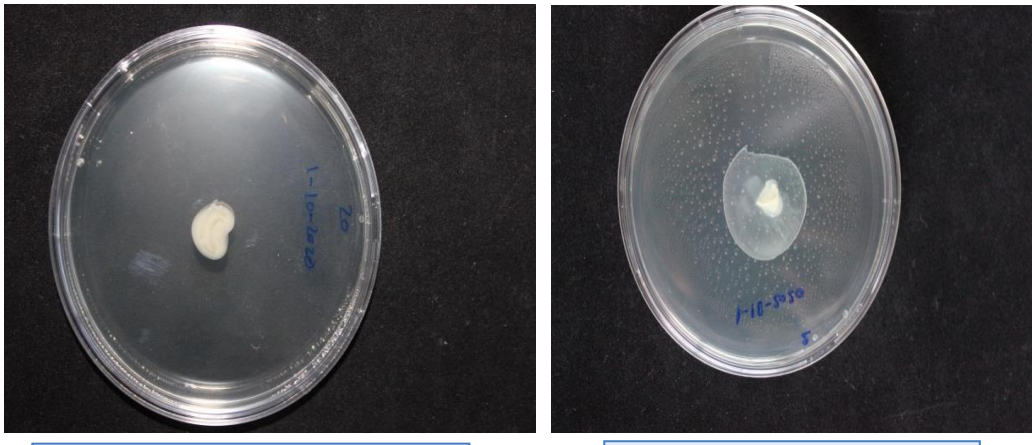

Fig. (1): Photograph of one Rhizobial isolate as representive of all tested isolates. Rhizobial isolates showed motile on solid YEM plates incubated at 28°C and examined after 24 and 48 hours (a) incubation at 28°C for 24 hrs, (b) incubation at 28°C for 48 hrs.

Table (2): Response of rhizobial isolates to Gram and Fucshin stain.

Isolates	Fuchsin stain (rod shape)	Gram stain
1	+	-ve
2	+	-ve
3	+	-ve
4	+	-ve
5	+	-ve
6	+	-ve
7	+	-ve
8	+	-ve
9	+	-ve
10	+	-ve
11	+	-ve
12	+	-ve
13	+	-ve
14	+	-ve
15	+	-ve
16	+	-ve
17	+	-ve
18	+	-ve
19	+	-ve
20	+	-ve
21	+	-ve
22	+	-ve
23	+	-ve
24	+	-ve
25	+	-ve

Fig. (2): Rhizobial tested isolates that stained by fuchsine showed to be rod in shape as it appeared under (10X100) light microscope, and it is shown in Table (2).

Table (3): Ability of tested *Rhizobium leguminosarum* isolates to produce acid or alkli.

Isolate	color
1	Yellow
2	Yellow
3	Yellow
4	Yellow
5	Yellow
6	Yellow
7	Yellow
8	Yellow
9	Yellow
10	Yellow
11	Yellow
12	Yellow
13	Yellow
14	Yellow
15	Yellow
16	Yellow
17	Yellow
18	Yellow
19	Yellow
20	Yellow
21	Yellow
22	Yellow
23	Yellow
24	Yellow
25	Yellow

Table (4): Ability of tested rhizobial isolates to use various sugars in growth media as sole carbon source .

Isolate	Sucrose	Sorbitol	Maltose	Glucose	Lactose	Mannose	Fructose	Mannitol (control)
1	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+

Data presented in above Figure (3) clearly showed that nine isolates (1,3,5,13,4,16,18,23, and 25) have the ability to grow at highest values at all tested pH from 5 to 8. On the other hand, bacterial isolates numbers 11,12 and 21 showed the least ability to grow at all pH.

Gebremoriam and Assefa, (2018) and Melak *et al*, (2018) published results that agreed and coincides to our obtained results. These tested isolates were able to tolerate pH ranging from 4 to 9 and some of them were not able to tolerate lower pH ranges (4 to5).

Ability of 25 tested isolates to grow on different NaCl concentration

Data presented in Figure (4) showed the ability of all tested *Rhizobium leguminosarum* to grow on different salt concentration from 0% to 4%. Data showed a vast variation of the tested isolates on growth in presence of 0.5% NaCl. Isolate 3 showed 281 colonies whereas isolate 19 showed 3 colonies. Isolate 1 showed the capacity to grow at higher colonies number over all tested NaCl concentrations ranging from 280 at 0.5% to 101 at 4% NaCl. It is clear from Figure (4) that there is a vast variation between tested rhizobial isolates in their capacity to tolerate various NaCl concentrations. Isolate number eleven showed the least numbers of colonies at various NaCl concentrations as it appeared to show 11,9,6, and 10 colonies for 0.5%,1%,2% and 4% respectively. Isolate number 18 showed the highest colony numbers to be tolerant to various NaCl concentrations. It showed 248,229,187, and 103 at 0.5%,1%,2%, and 4% respectively.

Data presented in Table (5) clearly show the ability of all tested rhizobial isolates to hydrolyse starch. However, those isolates were not able to hydrolyse urea, except isolates number 4 and (16).

PCR amplification and sequencing of 16s rRNA gene as universal gene for identification of bacterial isolates

We prepared total DNA from all studied isolates, moreover we amplified 16s rRNA gene using universal primers. As shown in Figure (5) all tested isolates showed same molecular weight for 16s rRNA gene as appeared in agarose gel to be about 1500bps. In fact, the obtained size band have been reported before (chen *et al*, 2018) and (Hussein, *et al* 2016). We have to mention that the obtained size we got in our experiment (1500) was shown by some authors and others showed a little bit difference as 1370bps Yuan *et al* (2016). Efstathiadu *et al* (2020) reported gene size to be 1303bps, and Zhang *et al*, (2022) that 16s rRNA gene sequence to be 1269 base pairs in Rhizobial strains isolated from China.

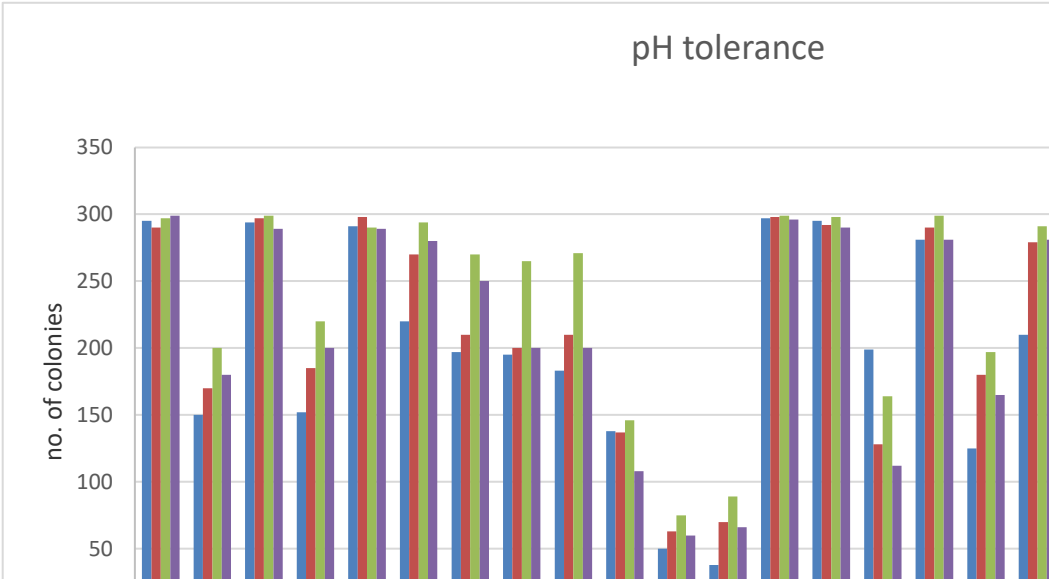

Fig. (3): Ability of 25 tested Rhizobium isolates to grow at various pH (5,6,7, and 8).

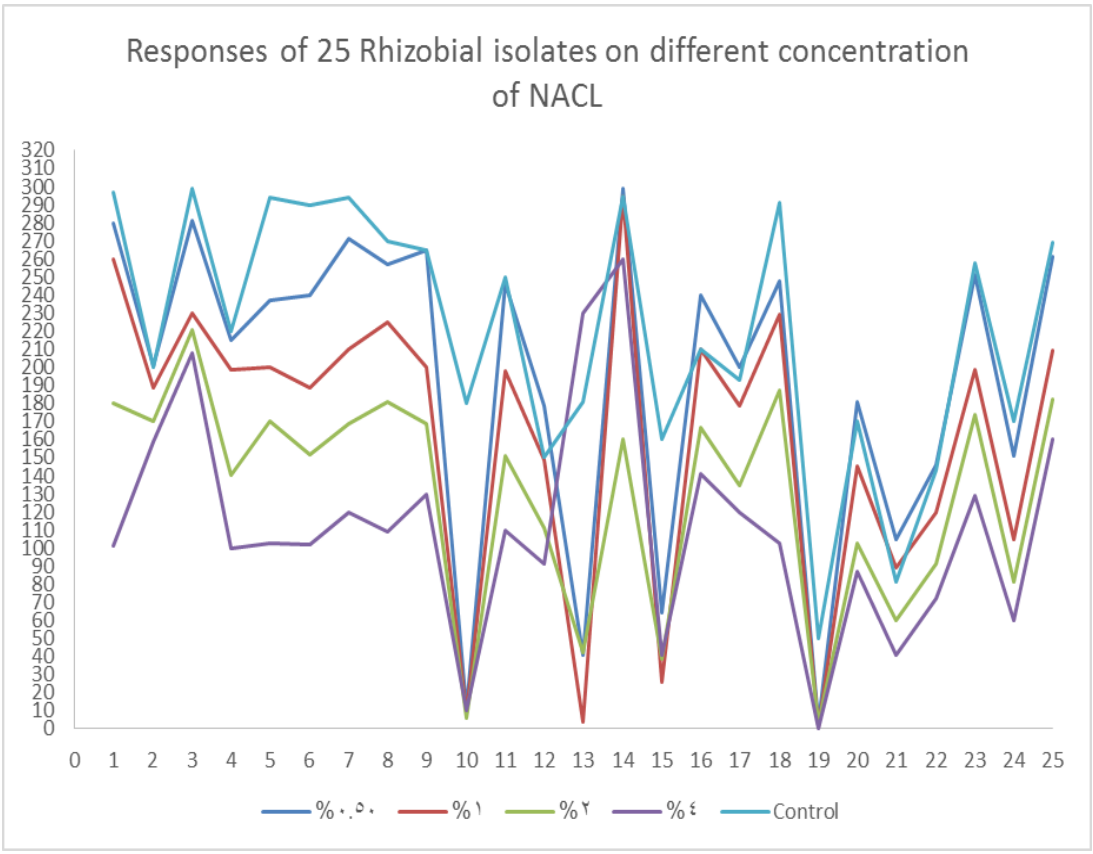

Fig (4): Ability of 25 rhizobial isolates to grow at various NaCl concentrations (0.5%,1%,2% and 4%).

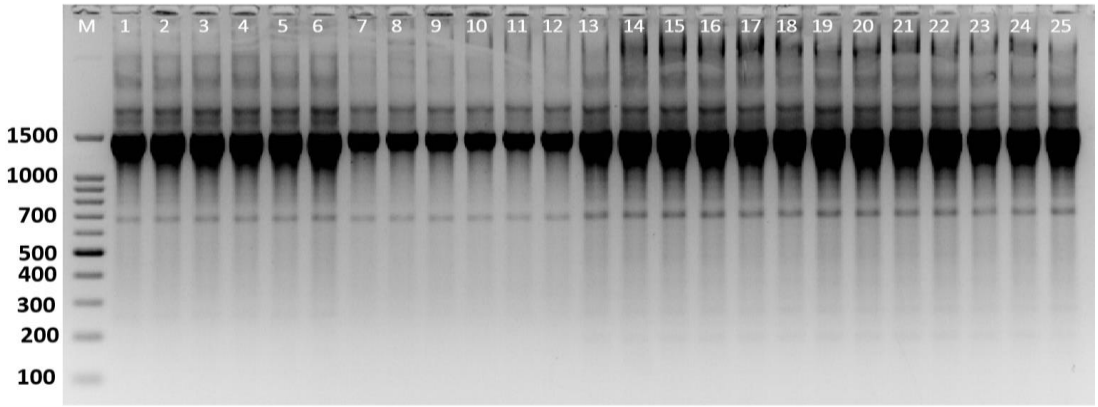

Fig (5): Genetic diversity of all tested rhizobial isolates was detected upon using 16s rRNA gene.

Table (5): Ability of 25 tested *Rhizobium leguminosarum* isolates to hydrolyze of starch and urea.

Isolates	Starch hydrolysis	Urea hydrolysis
1	+	-
2	+	-
3	+	-
4	+	+
5	+	-
6	+	-
7	+	-
8	+	-
9	+	-
10	+	-
11	+	-
12	+	-
13	+	-
14	+	-
15	+	-
16	+	+
17	+	-
18	+	-
19	+	-
20	+	-
21	+	-
22	+	-
23	+	-
24	+	-
25	+	-

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التعريف المورفولوجى ، البيوكيميائى والجزيئي لـ ٢٥ عزلة من بكتيريا الرايزوبيوم ليجيومينوزارم من نبات الفول البلدى في محافظة المنوفية، مصر

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الملخص العربى

جمعنا عقد جذرية من نباتات الفول البلدي من تسع مناطق مختلفة في محافظة المنوفية .تم عزل ٢٥ عزلة من الفول المصري. حددنا الخصائص المورفولوجية على عزلاتنا المختبرة (الحركة وصبغة فوكسين وصبغة الجرام) والخصائص الكيميائية الحيوية (إنتاج الحمض أو القلويات، واستخدام مصادر الكربون المختلفة، وتحمل الأس الهيدروجيني وكلوريد الصوديوم، وتحلل النشا، والتحلل المائي لليوريا)، وكذلك الخصائص الوراثية من خلال اكثار جين 16s rRNA. كانت جميع العزلات المختبرة متحركة وعصوية وسالبة لجرام وغير قادرة على امتصاص لون الكونغو الأحمر. أظهرت نتائج الاختبارات البيوكيميائية أن جميع العزلات المختبرة منتجة للحمض، سريعة النمو، لها قدرة على البقاء على قيد الحياة بتركيز كلوريد الصوديوم يتراوح من ٠,٥ إلى ٥٪ .كل منهم قادر على النمو في وجود مدي واسع من الأس الهيدروجيني (٥ إلى ٨) . وأظهرت جميع العزلات نفس الوزن الجزيئي لجين 16s rRNA والذى كان يساوي حوالي ١٥٠٠ زوج من القواعد في كل العزلات.

الكلمات المفتاحية:*Rhizobium leguminosarum* ، التعريف المورفولوجي، البيوكيميائي ، الفول البلدي ، جين 16s rRNA.