Nodule formation efficiency evaluation and *nifH* gene detection of Rhizobium species isolated from Egyptian soil

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ABSTRACT

Nitrogen Fixing bacteria have a major role in supplying plants with their nitrogen requirements. Fifty bacterial strains were isolated from the soil at Billay, Al-Santah, Gharbia governorate, Egypt, which had been previously cultivated with Egyptian Faba bean. They were classified into eight groups based on the morphological and biochemical tests. All groups have been tested for two approach, firstly effective Rhizobium inoculation on different hosts of legumes, secondly detection of nifH gene based on three different nifH gene primers. The results showed that the group number 5 has been positively formed nodulation on Egyptian clover roots, nifH gene is positively detected in Rhizobium isolates (5, 10, 12, 21, 22). This study recommend that the Rhizobium has intensive role as a biofertilizer in soil fertilization.

Keywords: *Rhizobium*; Nitrogen fixation; Biofertilizer.

INTRODUCTION

Nitrogen is an important plant alimentary for the optimal outgrowth and yield of major agricultural crops. Regarding crop productivity, microbial inoculants may supplement N-requirement and minimize reliance on costly synthetic N-fertilizer. Soil microorganisms' ability to fix atmospheric nitrogen is a key characteristic in encouraging plant growth and improving agricultural output. Microorganisms in the soil are the connecting between aboveground and underground ecosystems, which have a significant role in the management of soil environmental processes, the processing of organic material in the soil, and boost of nitrogen and carbon cycles (Yu et al., 2018).

Roots of all plant are the finest place which microbial communities gathering (Turner et al. 2013) . Phenotypic traits like structure of the roots may have an effect on microorganisms' growth and propagation. Primary metabolites like amino acids or organic acids can be excreted by the roots by Vives-Peris et al. (2020) , which have a significant function in forming the rhizosphere ecosystem through altering the chemically properties for soil around the roots are considered substrates for the growth of different microorganisms in the soil (Hu et al. 2018) . In accordance with the nutritional state of plants, growth phase, as well as its location in time and place , the construction of the root exudates varies both quantitatively and qualitatively (Backer et al. 2018) . Strong selection pressures caused by these secretions in the rhizosphere cause plants to select particular soil microorganism communities (Huang et al. 2014) .

Unfortunately, plant species cannot transfer the atmospheric di nitrogen to ammonia and directly use it for their growth. About 79% of the total atmospheric gases is the nitrogen as the greatest present gas in the Earth's atmosphere. Nitrogen may be found in amino acids, proteins, and a variety of other chemical molecules, and it is a necessary component for all forms of life (Berman-Frank et al. 2003) . Nitrogen-fixing microbes represent the only environmental source of biological nitrogen in the world, which enzymatically convert nitrogen to forms that can be used by plants like NH4+ or NO3–. and most of known nitrogen fixing bacteria have been uncultured in the laboratory yet (Zehr et al. 2003 ; Gaby and Buckley ,2012) . The atmospheric nitrogen can be fixed by some prokaryotic microorganisms from the gaseous form in the atmosphere Rubio and Ludden (2002), including (1) symbioses between legumes plants and bacteria, (2) symbioses between plants or fungi with cyanobacteria, (3) free-living bacteria in environment and (4) lightening-related abiotic reactions (Shridhar ,2012) .

Nitrogen fixation which is performed by the nitrogenase enzyme enzymes is an important process to the biological activity of soil, several subunits of which are encoded by the genes nifH, nifD, and nifK as described in (Rubio and

Ludden ,2002) ; Mohammed et al. 2018) . Bacteria like Rhizobium, Azotobacter, Azospirillum, SinoRhizobium and MesoRhizobium are well known for their ability to Nitrogen Fixing (González-López et al. 2005 ; Emtiazi et al. 2007) .

Both plants and bacterium can live separately but the interaction benefits both. It has been advertised that nitrogen fixation generates as much as 25 percent of total nitrogen in plants. Nitrogen-fixing microorganism activity is primarily dependent on high quantities of carbon compounds and low levels of combined nitrogen. Carbohydrates are derived either directly from photosynthesis (as in the case of cynobacteria) or indirectly through the decomposition of organic waste in the soil. The plant roots secrete chemicals into the soil that help bacteria grow and fix nitrogen in the rhizosphere around plant roots (Johnston *et al.* 2007) . The Rhizobia Bacteria (which includes the genera *Rhizobium, Bradyrhizobium, Mesorhizobium,* and *Sinorhizobium*), are distinguished by their capacity to form nodules on the legume roots. Inside nodules, the bacteria transformed into another form called bacteroid which can fix nitrogen from atmospheric to ammonia. This symbiosis relationship support the legumes with a personalized nitrogen source and, in return, the bacteria obtain a carbohydrate (Johnston *et al.* 2007 ; Mahmud *et al.* 2020) . This study aims to the isolation of some protentional nitrogen fixing bacteria from the Egyptian soil and the identification of these bacterial isolates depending on the morphological and biochemical characteristics and their capacity to form nodules on some legumes and the use of a particular PCR method.

MATERIALS AND METHODS

Soil Sampling; site, collection, and preparation

The study site was located in Billay, Al-Santah, Gharbia governorate, Egypt, (30°50'02.9"N 31°09'01.4"E) where the soil samples were collected from rhizosphere soil, any leaf debris or non-soil material that might end up in sample were brushed away, then the samples were immediately transported to the laboratory after collection and stored at 4oC. For soil extraction preparation the samples were mixed, with avoiding contaminating soil with skin flora or other lab environment organisms.

Three different samples of soil was weighed (1 gram) and 100 ml of sterile water was added to each in a sterile 250 ml flask with magnetic stirrer for at least 15 min, Then the soil sample suspension was let until the larger particulate matter settles to the bottom some of the supernatant was transferred into a new labeled sterile 50 ml conical tube, then used for bacterial cultural on Yeast extract mannitol agar media (YEMA) which compose of Yeast extract: 1.0 g, Mannitol: 10.0 g, MgSO4, 7H2O: 0.2 g, K2HPO4: 0.5 g, NaCl: 0.1 g, Distilled water: 1 liter, and Agar: 15 g. The medium was prepared and was autoclaved at 15 psi and 1210 C for 20 minutes.

Rhizobia isolation and purification

The soil was serial diluted.100µL of every dilution and the crude soil extract were transferred on YEM agar plate. The plates were incubated at 28°C for 24 hours, 50 different single colonies were selected, and cultivated on slant medium.

Morphological and Biochemical *Characterization of the isolates*

The selected colonies were characterized morphologically include colony shape, size, color, and capacity to change pH, as well as biochemical characteristics include lactose utilization and fermentation, and differentiation media by modifying the base media (YEMA) with the addition of several types of dyes as indicators such as Congo red, Bromothymol Blue, and Gram stain reaction. MacConkey agar was used to show the differentiation of lactose fermenting from lactose non-fermenting bacteria.

The intrinsic antibiotic resistance study was carried out to identify sensitivity or resistance of local isolates against different antibiotics which were listed in table 1. The selected colonies were tested for antibiotic (sensitivity/ resistance) by disc diffusion on Mullar-Hinton agar medium, antibiotics discs were placed equidistantly at the center of the 9 cm plates and then incubated overnight at 28°C. The resistances or sensitivity of isolates to antibiotics were determined by observation of the inhibition zone around the discs (Beshah ,2019) .

Nodulation test

Seeds of *vicia faba, trifolium alexandrinum, Pisum sativum* and *Phaseolus vulgaris* were surface sterilized with 3% sodium hypochlorite

solution for 5 min, then rinse many times with sterile distilled water, and were soaked in sterile water for 8 hr. Five seeds were distributed on the surface of planting bags containing sterile (pittmoss, perlite, sand) with percentage 2:1:1. The young seedlings will inoculate by applying 1.5 ml of isolate culture groups which were listed in table 3 to the root zone, then arrange randomly in greenhouse at 25/23°C d/n, and a photoperiod of 16 h. The culture will irrigate regularly with distilled water and N-free nutrient solution was added once a week (Kebede *et al.* 2022) .

NifH primer, and PCR amplification

Based on nodulation test, the bacterial isolates group that formed nodules on legumes roots were used in PCR reaction to amplify *nifH* gene as a gene marker for molecular identifying of rhizobium isolates using three *nifH* gene primers (Table 2) which were examined in silico for the recoverability of our genes based NCBI blast primer tool as followed by (Abo-Elwafa *et al.* 2018)

. *NifH* gene was amplified by PCR using three *nifH* primers for Group 5. The PCR reaction mixture (25 µl volume) contained 12.5 µl GoTaq® Green Master Mix (Promega, Madison, Wi, USA), 2 µl Forward primer (10 pmol/ µl), 2 µl Revers primer (10 pmol/ µl), on bacterial colony as DNA template, 10 µl Water nuclease free. The PCR program for three primer was 5 min Initial denaturation at 95°C, then 35 cycles of 1min denaturation at 94°C, 1min at (53, 54, 55, 56, 57, 58) were tested for annealing; 2min extension at 72ºC, and 7min final extension at 72ºC. For fragment size comparison: 5 µl of amplified genes and 5 µl of 100bp DNA ladder from Thermo Scientific Gene were migrated on 1.5% (wt/vol) agarose gel containing ethidium bromide (0.5 µg/ml) and visualized under UV light (Dual–Intesity Trans Illuminator).

RESULTS AND DISCUSSIONS

Phenotypic characterization of Rhizobium isolates on YEMA and Congo red.

The figure 1.A showed that on the basis of morphological characters, when bacterial isolates were grown in YEMA media, the colonies appear of circular and viscously with smooth edges and different colors between ivory, cream and yellow, and milky (white) to watery translucent appearance on YEMA medium except the two isolates (9, 23) have yellow color, which as listed in table 3 and 4.

This viscous appearance is due to the ability of these bacterial colonies produce carbon exopolysaccharides in rich media, and after many days of colonies growth in YEMA medium, the colonies viscously substance on the cover of the dish even if it is stored at 4oC. Alhayale and Al-Shakarchi (2021) , as shown in figure 2 (B) The purity of the rhizobia isolates was screened by adding Congo red in YEMA medium.The results show that all isolates have pink or light pink color except the isolates (19, 23, 39) have a red color, which as listed in table 3 and 4.

Most of Rhizobium isolates absorb the dye only weakly to appear in light pink whereas others will absorb strongly. these results agreed with Hewedy *et al.* (2014) ; Abo-Elwafa *et al.* (2018) ; Robledo *et al.* (2012) they mentioned that the phenotypic characterization of Rhizobium strains was rod-shaped gram negative, have a capability to form nodule on appropriate host plant legume, and white colony on YEM media containing Congo red stain.

Differentiation of fast growing and slow growing isolates

The results show the ability of 33 isolates to change the color of media to yellow. In comparison, 17 isolates change the media color to blue or do not make a change in media color as listed in Tables 3 and 4. Bromothymol blue dye was added to yeast Mannitol Agar (YEMA) media to classify the fast-growing isolates which produce acid in media from slow-growing isolates which produce alkali. The fast-growing isolates need about 2 days to produce an acidic reaction, which can be observed by changing the media color from green to yellow. In contrast, the slow growing isolates take more than 4 days to produce alkaline endpoints, which can be observed by changing the media color to blue from green, or without changing the media color as shown in figure 2.A. Pervin *et al.* (2017) ; Hamza and Alebejo (2017) , while the Figure 3.B showed the original green color in control plate 1 and show the difference of changing the color to blue in plate 2 which contain most of slow grower isolates and changing the color to yellow in plate 3 which contain most of fast grower isolates, while the plate 4 the color turning to clear yellow Because it contains fast-growing strains only.

MacConkey Agar Differentiates Lactose fermenters and non-fermenters.

The results showed the ability of 36 isolates to change the color of MacConkey media to red or pink, while 14 isolates don't make changes in media color as listed in Tables 3 and 4. MacConkey agar was used to isolate the gram-negative bacteria and show the differentiation of lactose fermenting from lactose non fermenting bacteria. As shown in figure 4: Lactose fermenting isolates grow as red or pink which may be surrounded by a zone of acid precipitated bile, and the red color is come from production of acid from lactose, while the lactose non-fermenting isolates are colorless and transparent and typically don't make change in appearance of the medium, which agrees with Téllez-Sosa *et al.* (2002)

Grouping the bacterial isolates

After filtering morphological and biochemical characteristics using Excel software. The fifty isolates were classified into 8 different groups as shown in Table 3, Where shows the availability of rhizobium traits in seven groups, three of them are fast-growing and four are slow growing, with the exception of group number 8 as appeared in yellow on the YEMA medium and in red on Congo red stain.

Antibiotic-resistance patterns

As shown in table 4 most of isolate show high resistance to the antibiotics (Ampicillin, Penicillin, Erythromycin, Azithromycin) and show highly sensitive for the antibiotics (Cefepime, Oflaxcin), while show different response for the other selected antibiotics.

Nodulation test:

The test showed that a group number five was able to form nodules on the Egyptian clover roots as shown in figure 4, which was later subjected to a PCR test to detect the presence of the gene *nifH* gene to confirm which isolates within this group formed nodules. On the other hand, the non-inoculated plants couldn't form any nodules on their roots, and suffered from nitrogen shortage, and had a yellow color in comparison to the green color of inoculated plants.

Amplification of *nifH* gene and visualization

Based on molecular biology tools, the result of genotypic analysis verified that the *nifH* gene is present in Rhizobium isolates (5, 10, 12, 21, 22). This result is consequently parallel with Laguerre *et al.* (2001) results which they mentioned that they achieved the same result by design degenerative primers that mentioned in table 3 to amplify *nifH* gene to be used in Rhizobium identification, which as shown in the Figure 5 the result of PCR using *nifH*, *nifH1*, and *nifH2* degenerative primers to amplify *nifH* gene with different size from different isolates as the *nifH* primer amplified fragment 780bp from isolate number 22, and the *nifH1* primer amplified fragment 660 bp from isolates number 5 and 21, and the nifH2 Primer amplified fragment 308 bp from isolates number 10 and 12, with annealing temperature 57 which had been the best of different temperatures were tested.

Agricultural technologies enabled a green revolution in the middle of the twentieth century, but at a significant environmental cost, contributing to global pollution, adverse climate change, and biodiversity loss Gu *et al.* (2016) . Biotechnology and microbiology work together to offer a broad field of research for increase crop quality, crop production, and the sustainability of current systems to generate more and better-quality agricultural goods using genetically modified organisms (GMOs) and transgenic crops. Microbial-Biotechnology has played an important part in sustainable agriculture in a variety of ways, including biofertilizers, bioherbicides, biopesticides, bioinsecticides, and so on. Microbial-Biotechnology minimizes pesticide dependency in sustainable agriculture by managing biotic and abiotic stressors.

Microorganisms in Rhizosphere play an essential role in agricultural sustainability across a variety of mechanisms Sasirekha and Srividya (2016) , including improving N2 fixation, increasing the availability of P, S, Zn, and Fe, controlling abiotic and biotic stressors, enhancing productivity, crop quality, and bioremediation Noble and Ruaysoongnern (2010) .

Microbial biotechnology aids agricultural production by reducing the need for chemical fertilizers, insecticides, herbicides, and other pesticides. It also preserves our environment safe and clean for future generations to enjoy. The advantage of microbial biotechnology is that it allows us to avoid using dangerous chemicals and wastes that harm natural resources and the

environment. Human action is causing big imbalances in the nitrogen cycles in soil as a resulting of increased chemical fertilizer use Shridhar (2012) .

The biological or non-biological factors which increase the quality and production of agricultural products and reduce the time of their growth are known as agricultural enhancers. The overuse of chemical fertilizers in agriculture has harmed the agricultural ecological system Sanchez *et al.* (2003) . Environmental contaminating increasing, like nitrate leaching and nitrous oxide emissions, causes more NO3- to be leached, possibly polluting groundwater. Increased NO3- levels may increase both direct and indirect N2O emissions into the environment Tanaka *et al.* (2006) .

CONCLUSION

Soil-plant microbial interactions have been proposed as a viable technique for improving nitrogen absorption and cycling. The usage of bio fertilizers is a viable option for improving agricultural conditions in Egypt and throughout the world. Biological fertilizers do not pollute the land or the environment and aid in the production of healthy foods Shridhar (2012) . The utilization of modern biotechnological tools conserves time, energy, and money by reducing the gap between researchers in Rhizobium identification and to overcome all obstacles in the use of biofertilizers as a safe alternative to chemical fertilizers.

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Table 1: The concentrations and mode of action of selected antibiotics.

No.	Antibiotics	Concentration	Mode of action
1	Cefoxitin	30 mcg	Cell Wall Synthesis
2	Cefepime	30 mcg	(Inhibition of the trans-peptidase enzyme)
3	Carbenicillin	100 mcg	
4	Amoxicillin	30 mcg	Cell Wall Synthesis
5	Ampicillin	10 mcg	(Inhibition of the trans-peptidase enzyme)
6	Penicillin	10 units	
7	Ofloxacin	5mcg	DNA
8	Streptomycin	10 mcg	Protein Synthesis Inhibitors (Inhibit 30s Subunit)
9	Erythromycin	15 mcg	Protein Synthesis Inhibitors
10	Azithromycin	15 mcg	(Inhibit 50s Subunit)

Table 2: *NifH* primer used in PCR reaction.

Primer	5 -3 Nucleotide sequence	Length	Reference
nifHF	TACGGNAARGGSGGNATCGGCAA	780 bp	Laguerre *et al.* (2001)
nifHR	AGCATGTCYTCSAGY TCNTCCA		
nifH1F	AATACRCTCGCYGCYCTBGT	660 bp	Abo-Elwafa *et al.* (2018)
nifH1R	TCGRTATTCYGCCGCCTGTT		
nifH2F	GCIWTYTAYGGNAARGG	308 bp	Gaby and Buckley (2014)
NifH2R	CCRCCRCANACMACGTC		

Table 3: The isolates culture Groups and their Morphological characteristics on different media.

No.	Groups	YEM	Congo red	BTB	MacConkey
1	14 – 26 – 27 – 28 – 38 – 48	Wight	Pink	Slow	Ferment lactose
2	8 – 15 – 16 – 25 – 29	Wight	Pink	Slow	Non-Ferment lactose
3	1 – 20 – 31 – 45 – 46 – 47 – 50	Wight	Pink	Fast	Non-Ferment lactose
4	3 – 4 – 11 – 13 – 30 – 32 – 33 – 34 – 35 – 37 – 40 – 41 – 42 – 44 – 49	Wight	Pink	Fast	Ferment lactose
5	5 – 6 – 10 – 12 – 18 – 21 – 22 – 36 – 43	Wight	Light Pink	Fast	Ferment lactose
6	2 – 17 – 24	Wight	Light Pink	Slow	Ferment lactose
7	7	Wight	Light Pink	Slow	Non-Ferment lactose
8	9 – 19 – 23 – 39	Non-Rhizobium			

Table 4: Antibiotic-resistance patterns for the isolates (1-50)

Isolates code	Cefoxitin	Cefepime	Carbenicillin	Amoxicillin	Ampicillin	Penicillin	Ofloxacin	Streptomycin	Erythromycin	Azithromycin	Isolates code	Cefoxitin	Cefepime	Carbenicillin	Amoxicillin	Ampicillin	Penicillin	Ofloxacin	Streptomycin	Erythromycin	Azithromycin
1	+	-	+	-	+	+	-	+	+	+	26	-	-	-	-	-	+	-	+	-	-
2	+	-	+	-	+	+	-	+	+	+	27	+	+	+	+	+	+	+	+	+	+
3	-	-	+	-	+	+	-	-	+	+	28	+	+	+	+	+	+	-	+	+	+
4	+	-	+	+	+	+	+	-	+	+	29	+	-	+	+	+	+	-	-	+	+
5	-	-	+	-	-	+	+	-	+	+	30	-	-	+	-	+	+	-	+	+	+
6	+	-	+	-	+	+	-	-	+	-	31	+	-	+	+	+	+	-	-	+	+
7	-	-	-	-	+	+	-	+	+	+	32	-	-	-	-	+	+	-	-	+	-
8	+	-	+	-	+	+	-	-	+	+	33	+	-	+	-	+	+	-	-	+	+
9	-	-	-	-	+	+	-	+	+	+	34	+	-	+	-	+	+	-	+	+	+
10	+	-	+	-	+	+	-	+	+	+	35	-	+	-	-	+	+	-	-	+	+
11	-	-	+	-	+	+	-	+	+	+	36	+	-	+	-	-	-	-	+	+	+
12	-	-	-	-	+	+	-	-	+	+	37	+	-	+	-	+	+	-	-	+	+
13	+	-	-	-	+	+	+	+	+	+	38	-	-	+	-	+	+	-	-	+	+
14	-	-	+	-	+	+	-	-	+	+	39	+	-	+	-	+	+	-	+	+	+
15	+	-	+	-	+	+	-	+	+	+	40	-	-	+	-	+	+	-	-	+	+
16	+	-	+	-	+	+	-	+	+	+	41	+	-	+	+	+	+	-	-	+	+
17	-	-	+	-	+	+	-	-	+	-	42	-	-	+	+	+	+	-	+	+	+
18	+	-	-	-	+	-	-	-	+	+	43	+	-	+	-	+	+	-	-	+	+
19	+	-	+	+	+	+	-	+	+	+	44	+	-	+	+	+	+	-	-	+	+
20	+	-	+	+	+	+	-	+	+	+	45	+	-	+	+	+	+	-	-	+	+
21	+	+	+	+	+	+	-	+	+	+	46	+	+	+	+	+	+	-	-	-	+
22	-	-	+	-	+	+	-	-	+	+	47	+	+	+	+	+	+	-	-	+	+
23	-	-	-	-	+	-	-	-	-	-	48	+	-	+	+	+	+	-	-	+	+
24	+	-	+	-	+	+	-	-	+	+	49	+	-	+	-	+	+	-	+	+	+
25	+	+	+	+	+	+	+	+	+	+	50	+	+	+	+	+	+	-	+	+	+

+: Resistant -: Sensitive

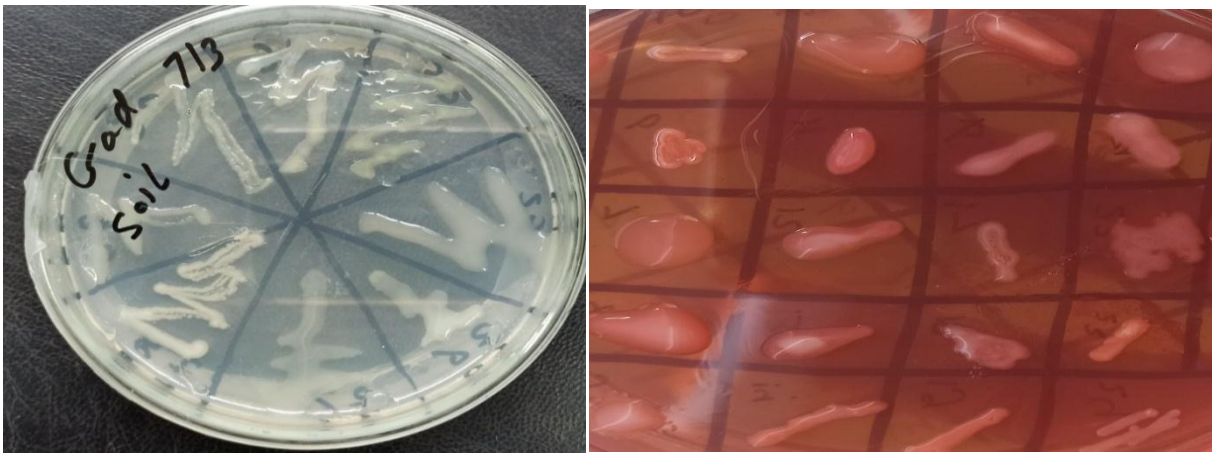

Figure 1: Growth of selected isolates (A) on YEMA and (B) on YEMA with Congo red.

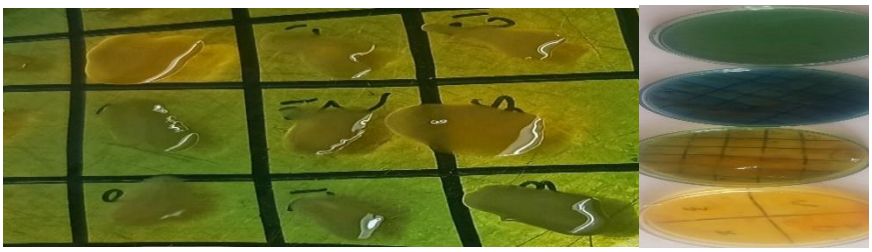

Figure 2: The Growth of selected isolates (A) on YEMA with Bromothymol blue and (B) show the color changing on media.

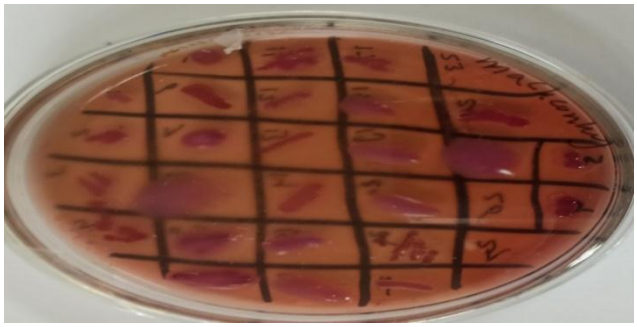

Figure 3: the Growth of selected isolates on MacConkey media

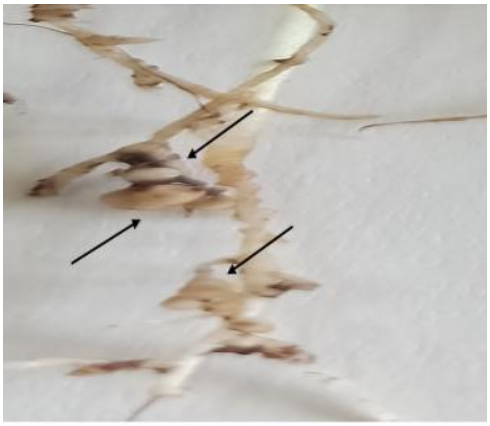

Figure 4: The nodules formation on Egyptian clover roots

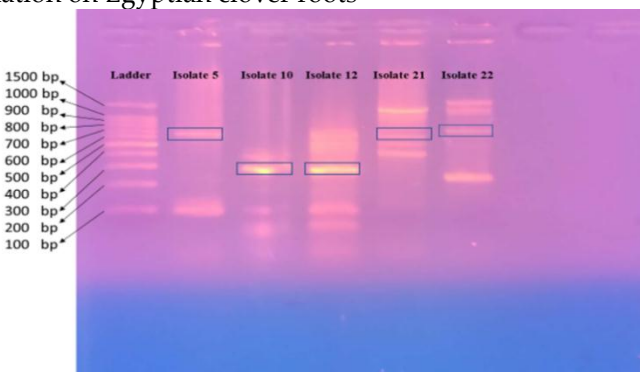

Figure 5: PCR amplified fragments using *nifH* primers, Ladder = 100bp DNA ladder. primer (*nifH*): amplified fragment 780bp from isolate number 22. primer (*nifH1*): amplified fragment 660 bp from isolates number 5 and 21. Primer (*nifH2*): amplified fragment 308 bp from isolates number 10 and 12.

تقييم كفاءة تكوين العقيدات والكشف عن جين nifH لأنواع الرايزوبيوم المعزولة من التربة المصرية

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الملخص العربي

تلعب بكتيريا تثبيت النيتروجين دورًا رئيسيًا في إمداد النباتات بمتطلباتها من النيتروجين. تم عزل خمسين سلالة بكتيرية من التربة في قرية بلاي، مركزالسنطة، محافظة الغربية، مصر، والتي سبق زراعتها بالفول البلدي المصري. ثم تم تصنيفهم إلى ثماني مجموعات بناءً على بعض الاختبارات المورفولوجية والكيميائية الحيوية. تم اختبار جميع المجموعات من أجل نهجين، أولاً تلقيح رايزوبيوم الفعال على مضيفات مختلفة من البقوليات، وثانيًا الكشف على مستوي البيولوجيا الجزيئية عن جين nifH بناء على ثلاث بادئات جينية مختلفة. باستخدام جهاز تفاعل البلمرة المتسلسل (PCR) أظهرت النتائج أن المجموعة البكتيرية رقم خمسة قامت بتكوين عقيدات على جذور البرسيم المصري، وكذلك وجود جين nifH في عزلات الرايزوبيوم رقم (5، 10، 12، 21، 22). هذه الدراسة توصي بأن يكون لبكتريا الرايزوبيوم دور مكثف كسماد حيوي في تخصيب التربة.

الكلمات الاسترشادية: رايزوبيوم، تثبيت النيتروجين، سماد حيوي.