" Isolation and Characterization of Local strains of *Rhizobium leguminosarum* bv. *trifolii* "

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Abstract

This study included isolation and characterization of seven strains of *Rhizobium leguminosarum* bv. *Trifolii* from root nodules of clover plants. These strains as follows: WS1, WS2, WS3, WS4, WS5, WS6 and WS7. Clover plants were isolated from different agroclimatic regions of Mosul-Iraq. Symbiotic characteristics of clover plants inoculated with the fore-isolated strains of rhizobia revealed that the strain WS4 was the best, which has the symbiotic efficiency 15.6. This strain had been chosen for further studies; such as the effect of different incubation periods on growth of WS4 strain and pH changes of MSY medium. Three-day after incubation was the best with average optical density 1.83, when wavelength was 545 nm. Intensive incubation period resulted in decreasing the average values of optical density. Antibiotic resistant studies showed the ability of strains in resisting chloramphenicol, rifampicin, streptomycin, ampicillin and naldixic acid, whereas it was sensitive to tetracycline. The strain WS4 was able to grow on RMM. Study of production of cell surface molecules revealed disability of this strain to produce β (1 ⟶ 3) cyclic glucans, whereas it had the ability to produce the exopolysaccharide and cellulose fibrilis. SW4 was motile, this indicating its ability to produce β-(1 ⟶ 2) cyclic glucans.

Introduction

Rhizobia live freely in the soil, but when they approach the plant root, they are assumed to be an alien invader. The rhizobia induces the formation of specialized structures, called nodules, on the roots of host plants. The reduction of atmospheric nitrogen takes place in the nodules [1]. Many factors can reduce survival and growth of rhizobia in the soil and inhibit rhizobia-legume symbiosis such as nutrient deficiency and strain variation [2,3]. There is a need to select strains for specific geographic, soil and environmental regions and, ideally, for individual varieties of a given legume species [4]. *Rhizobium* strains must be selected on the bases of their competitiveness and efficiency in N_2 fixation upon nodule formation[5]. During rhizobium-legume interactions, cell surface molecules such as extracellular polysaccharides, β (1 ⟶ 3) cyclic glucans, β (1 ⟶ 2) cyclic glucans and cellulose fibrils showed important role [6]. Selecting efficient nitrogen fixer strains symbiotically is very useful can be introduced into soils of respective stress where competition from naturally occurring rhizobia is poor. In the present work isolation and characterization of local strains of *Rhizobium leguminosarum* bv. *trifolii* was done. The strain that showed the best symbiotic interactions had undergone the detailed work.

Materials and Methods

1. New strains of Rhizobia

Rhizobium leguminosarum bv. *trifolii* strains were isolated from root nodules of clover plants. The plants were collected from different agroclimatic locations of Mosul- Iraq.

2. Host plant cultivar

The seeds of clover (*Trifolium alexandrium*) plants were obtained from local market.

3. Media

3.1. Mannitol Salt Yeast Extract (MSY) Medium [7]. (g/l) : mannitol, 10; yeast extract, 0.2; K_2HPO_4, 0.2; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.1 and $CaCl_2.2H_2O$, 0.05, pH was adjusted to 6.8. This medium was used for growing, maintenance and antibiotics resistance test of rhizobial strains. This medium also used to study the effect of different incubation periods on growth of isolated strains and changes in pH.

3.2. Nitrogen free (NF) Plant Medium [8], (g/l) : $CaCl_2$, 132; $MgSO_4.7H_2O$, 120; KH_2PO_4, 100; $Na_2HPO_4.2H_2O$, 150; Fe-citrate, 0.05; $MnSO_4.2H_2O$, 0.11; $CuSO_4.5H_2O$, 0.025; $ZnSO_4.7H_2O$, 0.28; $CoCl_2.6H_2O$, 0.024; H_3BO_3, 0.062 and $NaMoO_4.2H_2O$, 0.024. pH was adjusted to 6.0. This medium was used for authentication and symbiotic response of local isolated strains.

3.3. Rhizobial Minimal Medium (RMM) [9] :Solution A, (g/l) : $Na_2HPO_4.12H_2O$, 0.45; $(NH_4)_2SO_4$, 2.0; $FeCl_3$, 2.0; $MgSO_4.7H_2O$, 0.1 and $CaCl_2.2H_2O$, 0.04. This solution, after adjusting its pH to 7.0, was autoclaved.

Solution B: This solution, contains glucose (20 %) in distilled water, was filter sterilized.

To prepare 1.0 Liter of RMM, 10 ml of solution B was added to 990 ml of solution A. This medium was used for testing auxotrophs of rhizobial isolated strains as well as utilization of sugars and dicarboxylic acids.

3.4. Yeast Extract Mnnitol (YEM) Medium [10], (g/l) : mannitol, 10; yeast yxtract, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2 and NaCl, 0.1, pH was adjusted to 6.8. This medium was used for testing succinylated exopolysaccharides, cyclic β-(1⟶ 3) glucans and cellulose fibrils production.

3.5. Tryptone Yeast Extract (TY) Medium [11], (g/l) : tryptone, 5.0; yeast extract, 3.0 and $CaCl_2.2H_2O$, 0.12, pH was adjusted to 7.0.. TY swarm plates contained 0.3 % (w/v) agar [6]. This medium was used for testing motility.

4. Maintenance of rhizobial strains

Purified local isolated strains of *Rhizobium leguminosarum* bv. *trifolii* were streaked on slants of

MSY solid medium. After a growth period of 24-48 hours at 28±2 ºC, slants were stored at 4 ºC in a refrigerator. Subculturing of rhizobial strains was done every two months.

5. Supplements to media

5.1. Antibiotics

The antibiotic resistance patterns were determined. Six antibiotics were used in this study. Stock solutions of chloramphenicol (Cm), rifampicin (Rif), tetracycline hydrochloride (Tc) and ampicilin (Am) were prepared in ethanol. Streptomycin sulphate (Sm) solution was prepared in distilled water while naldixic acid (Nal) was dissolved in 0.05 N NaOH. Antibiotic solutions were sterilized by passing them through 0.45 µm Millipore membrane filters and stored at 4 ºC. Different concentrations of antibiotic were added to the autoclaved medium after cooling it to 50 ºC, just before plating. Resistance to the fore-mentioned antibiotics was observed by spot test on MSY medium containing different concentrations of these antibiotics. Maximum concentration of antibiotics that was tolerated was determined [12].

5.2. Dyes

Aniline blue was added to test cyclic β (1 ⟶ 3) glucans production or calcoflour white for testing succinylated exopolysaccharides was added to the YEM medium at the rate 0.02 % (w/v), while congo red for testing cellulose fibrils production was added to the same medium at 0.1mg/ml final concentration. Every reagent was added to the medium before autoclaving [6].

6. Isolation of rhizobial strains from their host plant

Vencent [10] procedure was followed to isolate rhizobial strains from the berseem root nodules. Three to four pinkish nodules were washed in distilled water and exposed to 70 % (v/v) ethanol for 30 sec and to 5% NaOCl for 4 mins. These nodules were then washed ten times with sterile distilled water. The nodules were then crushed aseptically in 1.0 ml sterile saline (0.85 % w/v NaCl) with a sterilized glass rod. Suspension (0.1 ml) was spread on MSY solid medium and the plates were incubated at 28±2 ºC for 2-4 days. Transparent mucoid or gummy colonies were picked for further purification.

7. Infection confirmation in host plant roots

Seeds of clover plants were sterilized as described by Zilli *et al*. [13] and transferred onto nitrogen free agar slants in 20 x 2.5 cm tubes. Two 2-days old seedlings in each tube were inoculated with 10^8 cells (suspended in sterile distilled water) of a particular rhizobial strain. The growth conditions for the plants were 2000 lux light, a photoperiod of 16 hrs, and a dark period of 8 hrs at 25 ºC. The induced nodules on roots of clover plants by *Rhizobium leguminosarum* bv. *trifolii* strains were noticed after three days to one week.

8. Symbiotic characteristics of berseem plants inoculated with a local isolated *Rhizobium leguminosarum* bv. *trifolii* strains

Symbiotic characteristics of clover plants inoculated by *Rhizobium* done by following the same steps in the confirmation test. The morphological features of plants were recorded six weeks after inoculation. For determining the dry plant shoot weight, the plant tops were collected and dried in an oven at 65 ºC for 72 hr and then weighed. Reisolation of bacteria from nodules was done to confirm the nodule occupancy by a particular strain. Symbiotic efficiency was estimated according to Herridge and Roughley's equation [14] as follows:

Symbiotic efficiency =

Treated shoot plant dry wt. - Uninoculated control shoot plant dry wt.

Noduledry wt.

9. Effect of different incubation periods on growth of WS4 strain and changes in pH of MSY broth medium

Twenty mililiter of MSY broth was inoculated with 0.1 ml of WS4 culture of 10^7 to 10^8 cells/ ml as described by Steinborn and Roughley [15]. Inoculation of WS4 strain was done in triplicate. The flasks were then incubated in a rotary shaker operating at 120 rpm for 5 days at 28±2 ºC. Cell densities and pH changes of the suspensions were recorded on intervals for 24 hrs spectrophotometrically at 545 nm and with pH meter, respectively [13].

10. Utilization of sugars and dicarboxylic acids by WS4 strain

Utilization of several sugars (arabinose, maltose, manose, mannitol and sucrose) and dicarboxylic acids (malic acid, aspartic acid and sodium succinate) was studied. Rhizobial strain WS4 was streaked on solid RMM (without glucose) supplemented with one of the fore-mentioned sugars or dicarboxylic acids. Incubation was done at 28±2 ºC for 5 days and after 48 hrs of incubation, bacterial growth was observed daily [16].

Results and Discussion

1. Isolation of rhizobial strains from their host plant

Seven local strains of *Rhizobium leguminosarum* bv. *trifolii* were isolated from root nodules of clover plants. These strains are: WS1, WS2, WS3, WS4, WS5, WS6 and WS7. Sultan and AL-Safar [17] isolated twelve local strains of *Rhizobium leguminosarum* bv. *trifolii* from different agroclimatic regions of Ninavah province-Iraq, but no further studies done on these strains because they focused on *Sinorhizobium meliloti* NaCl tolerated strains. Simon [5] isolated 49 new strains of *Rhizobium leguminosarum* bv. *trifolii* he found high differences in each isolate's characteristics. He selected nine effective isolates.

2. Confirmation of infection of roots of clover host plant

Confirmation test of infection of isolated strains of *Rhizobium leguminosarum* bv. *trifolii* of roots of clover plants showed that these strains has the ability

to induce nodule formation on roots of clover plants, this result means that specific isolated strains enter in a interrelation with host plants.

3. Symbiotic characteristics of clover plants inoculated with rhizobial strains

Results of this study showed that the nodules induced by all the strains were pinkish in colour except WS5 and WS6 strains, which induced pinkish white nodules. These nodules were located on both primary and lateral roots except WS2 and WS5 strains, which induced nodules located on lateral roots only. All the strains induced the formation of cylindrical-shape nodules cylindrical in shape. Other symbiotic characteristics presented in Table (1). According the data recorded in this table, the maximum shoot length of clover plants was (20.9cm) when these plants inoculated with strain SW4, whereas WS5 strain resulted in plants with minimum mean shoot length (9.8cm). Mean shoot dry weight of plants inoculated with rhizobial strains ranged from 9.0 mg in WS6 to 22.5 mg in WS4 strain.

Table (1): Symbiotic characteristics of clover (*Trifolium alexandrium*) plants inoculated with local isolated strains of *Rhizobium leguminosarum* bv. *Trifolii*

Strain	Mean shoot length (cm)	Mean shoot dry weight (mg)	Mean no. of days to first nodule	Nodule characteristics		
				Mean no./plant	Mean dry wt. (mg) /plant	Sym. eff.
Cont.	6.1* ± 0.8	05.3 ± 1.1	-----	-----	-----	-----
WS1	14.4 ± 1.2	15.8 ± 0.7	12.2 ± 1.5	5.3 ± 0.8	1.1 ± 0.5	09.5
WS2	11.8 ± 0.6	14.4 ± 0.8	12.9 ± 1.4	5.4 ± 0.7	1.0 ± 0.6	08.8
WS3	17.7 ± 0.9	16.3 ± 1.1	10.5 ± 0.8	5.9 ± 0.9	0.9 ± 1.0	12.2
WS4	20.9 ± 0.3	22.5 ± 0.3	09.4 ± 1.9	6.2 ± 1.3	1.1 ± 0.9	15.6
WS5	9.8♦ ± 1.1	10.9 ± 0.6	15.1 ± 0.7	4.0 ± 0.7	1.0 ± 1.4	05.6
WS6	10.3 ± 0.9	09.0 ± 0.4	15.5 ± 0.5	3.8 ± 1.1	0.6 ± 1.2	06.1
WS7	20.1 ± 0.7	19.3 ± 2.2	08.1 ± 1.8	6.6 ± 0.5	1.2 ± 1.1	11.6

* Each value is mean of eight plants, Cont. = control (uninoculated plants),
♦ Significantly differences from the control (P< 0.05), ± = Standard deviation (S.D.), Sym. eff. = Symbiotic efficiency

Mean number of days to appear the first nodule varied from 8.1in SW7 strain to 15.5in strain WS6. Regarding to nodule characteristics, mean number of nodules per plant ranged from 3.8 in strain WS6 to 6.6 in strain WS7. The minimum mean nodule weight per plant (0.6 mg) was observed in strain WS6, whereas the strain WS7 produced the maximum value (1.2 mg) for this character. On the basis of Herridge and Roughley [14] equation the strain SW4 was found to be symbiotically most efficient (15.6). This strain was chosen for further studies. Kumar [12] found that symbiotic efficiency ranged between 4.1 to 12.5 when he isolated different strains of *Rhizobium leguminosarum* bv. *Trifolii* from Indian soils.

4. Antibiotic resistant profile of WS4 strain

Antibiotic resistance patterns of WS4 strain showed sensitivity to tetracycline and resistance to other studied antibiotics. The minimum antibiotic tolerance was for the streptomycin (60 µg/ml), and the maximum antibiotic tolerance was for the naldixic acid (180 µg/ml). The strain could tolerate rifampicin up to 70 µg/ml, whereas tolerance to ampicillin was up to 80 µg/ml. Multiple resistance to antibiotics explain existence of these strains in soil and then enter symbiotic relation with their host plant [18,13]. The antibiotic resistance may be helpful in further genetic analyses in this strain [19].

5. Effect of different incubation periods on growth of WS4 strain and changes in pH of MSY broth medium

Table (2) show that optical density increased with increasing the incubation periods and reached to a maximum (1.83) after three days of incubation. After this period, i.e. four days, optical density started decreasing and became 1.66 after five days incubation. Kumar [12] also found that optical density of *Rhizobium leguminosarum* bv. *trifolii* strains isolated from Indian soils decreased after 72 hrs incubation.

Final pH decreased from initial pH (6.8) to 5.1 after one day of incubation (Table 2). Increasing in incubation periods resulting in decreasing in pH, i.e. it reached to 4.5 after five days of incubation. Similar results obtained when Kumar [12] studied the effects of incubation periods on *Rhizobium leguminosarum* bv. *trifolii* strains isolated from Indian soils. It has been reported that during the growth of rhizobia in yeast extract/ sugar preparation, incomplete oxidation of sugars takes place which yields acid end products [20].

6. Utilization of sugars and dicarboxylic acids by WS4 strain

Results of this study showed that no change in the growth behavior of WS4 strain was detected when glucose in RMM was replaced by any one of the

other sugars (arabinose, maltose, mannose, mannitol and sucrose) or dicarboxylic acids (malic acid, aspartic acid and sodium succinate) as carbon source. Sridhar *et al*. [21] isolated different strains of fast growing rhizobia were able to utilize a wide range of carbon sources. Such these strains considered high competitive in comparison with other strains [2,18].

7. Test for growth of SW4 strain on Rhizobial Minimal Medium (RMM)

Results of this study showed that the SW4 strain was able to grow on RMM solid medium after 4-5 days incubation period. This result means there is no defect in any of biosynthetic pathways [22]. Such this strain is useful for genetic studies [3].

8. Test for the production of cell surface molecules by WS4 strain

Cell surface molecules production by the WS4 strain revealed that disability of this strain to produce β (1 ⟶ 3) cyclic glucans, whereas positive results could be obtained for the production of cellulose fibrils and succinylated exopolysaccharide (EPSI). Swamynathan and Singh[6] mentioned that the production of β (1 ⟶ 3) cyclic glucans found in *Agrbacterium tumefaciens* but not found in *Rhizobium*, also they mentioned the role of cellulose fibrils and succinylated exopolysaccharide in the *Rhizobium*-legume symbiosis. Motility test showed that the WS4 strain was motile. Production of β (1⟶ 3) cyclic glucan related with higher motility of rhizoial bacteria, this will be helpful to it in its competitive ability [6,23].

Table (2): Effects of different incubation periods and changes in pH of MSY broth medium on the growth of *Rhizobium leguminosarum* bv. *trifolii* strain WS4

	Incubation (Day)				
	1	2	3	4	5
Optical density	1.58* ± 0.05	1.61 ± 0.02	1.83 ± 0.01	1.70 ± 0.03	1.66 ± 0.04
Final pH	5.1 ± 0.03	5.0 ± 0.05	4.9 ± 0.03	4.8 ± 0.06	4.5 ± 0.01

* Each value is average of three replicates, ± = Standard deviation (S.D.)

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" عزل وتوصيف سلالات محلية لبكتريا *Rhizobium leguminosarum* bv. *trifolii* "

وجدان سالم قاسم

المعهد التقني ، الموصل ، العراق

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الملخص

تضمنت هذه الدراسة عزل سبع سلالات من بكتريا *Rhizobium leguminosarum* bv. *trifolii* من العقد الجذرية لنبات البرسيم (*Trifolium alexandrium*)، وهي كالاتي: WS1, WS2 ,WS3, WS4, WS5,WS6 و WS7. تم جمع نباتات البرسيم من مناطق زراعية مختلفة من الموصل/ العراق. أظهرت دراسة الصفات التعايشية لنباتات البرسيم اظهر ان السلالة WS4 هي الافضل حيث بلغت الكفاءة التعايشية 15.6. اختبرت هذه السلالة لدراسات تفصيلية اخرى، كدراسة تاثير مدد تحضين مختلفة على نمو السلالة WS4 وتغيير الاس الهيدروجيني لوسط MSY السائل. ان اعلى معدل كثافة ضوئية بلغت 1.83 عند طول موجي 545 نانوميتر بعد ثلاثة أيام من التحضين، اما زيادة مدة التحضين فقد ادى الى انخفاض معدل الكثافة الضوئية. اوضحت هذه الدراسة قابلية السلالة WS4على مقاومة بعض المضادات الحياتية مثل الكلورامفينيكول، الريفامبسين، الستروبتومايسين، الامبسلين وحامض النالديكسيك الا أنها كانت حساسة تجاه التتراسايكلين. تمكنت السلالة WS4 من النمو على وسط الرايزوبيوم الادنى كما اظهرت دراسة انتاج جزيئات سطح الخلية ان هذه السلالة لاتنتج كلوكونات β(1 ⟵ 3) الحلقية غير انها منتجة للبيفات السليلوز والسكر المتعدد الخارجي. لقد كانت السلالة المدروسة WS4 متحركة وهذا يدل على قابلية انتاجها لكلوكونات β (1 ⟵ 2) الحلقية.