IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 2 Ver. IV (Mar-Apr. 2014), PP 32-42
www.iosrjournals.org

Effects of pH on *Ensifermeliloti - Medicago sativa*Symbiosis

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**This paper is part of an M.Sc. thesis of the first auther.*

Abstract: *A total of ten Ensifermeliloti were isolated from root nodules of Medicago sativa L. (alfalfa) collected from different areas of Babylon province of iraq through the period of November-2012 to February-2013. The isolates were identified using polymerase chain reaction (PCR) technique, in addition to traditional methods.The PCR analysis showed that the isolates were harboured the nodbox 4 locus and the mucR genes which confirm that these isolates belong to E. meliloti.The symbiotic properties of the isolates atpH 5 – 10 were studied. It was found that the optimum pH for growing of Em1, Em3, Em4, Em6 and Em8 isolates was pH7 , while for the remaining isolates, represented by Em2, Em5, Em7, Em9 and Em10was pH8. All isolates were able to infect the root hairs and form nodules on the roots of alfalfa plants (Nod^+)at pH7,pH7.5 and pH8. The plants nodulated by these isolates were healthy and green (indicating that nitrogen was being fixed). However , the growth of isolates at pH6.5 (except for Em1 and Em8 isolates)and pH8.5 were unable to infect the root hairs and failed to elicite nodules on M. sativa (Nod^-).These plants were weak, stunted and became chlorotic six weeks after inoculation and resembled to the control (without inoculum) plants in all respects. These results indicate that the pH was significant factor and play essential role in the optimum nitrogen fixation .*

Keywords*:pH, Esifermeliloti ,Medicago sativa , symbiosis.*

I. Introduction

Sinorhizobiummeliloti offered a taxonomic challenge since many years which renamed recently to *Ensifermeliloti*[1]. This bacterium is use as a biofertilizer to increase the crop productivity, decrease the environmental pollution and improve the soil fertility to benefit the subsequent crop. Furthermore, the cost is so low that every farmer can afford using them, and these are the best alternatives to the inorganic (synthetic) fertilizers [2]. *E. meliloti*is able to enter into a symbiotic association with alfalfa plantwhich can be divided into three major stages: the first stage is preinfection or recognition which includes exchange of specific signal molecules (flavonoids and Nod factors) between *E. meliloti*and root hairs of *M. sativa*, the second stage is infection with concomitant nodule formation, in this stage the host cell wall is hydrolyzed and invasion tube called infection thread is induced which carries the invading bacteria towards the base of root hair, the cells of root cortex are mitotically activated and form nodule primodium then the bacteria released from infection thread into the cytoplasm of invaded host cells and be surrounded by host-derived peribacteroid membrane which controls the nutrient transfer between the symbionts then the divided bacteria differentiate into nondividingbacteroids (symbiosomes) , the last stage is nodule function, in this stage the symbiosomes be able to reduce atmospheric N_2 to NH_3 through the activation of the nitrogenase complex [3]. *E. meliloti*is a fast growing rhizobia that has a moderately small genome size of 6.7 million base pairs, the genome is still quite complex and is made up of three circular elements of DNA known as replicons, a chromosome 3.65 Mb and two megaplasmidspSymA 1.35 Mb and pSymB 1.68 Mb [4]. The alfalfa plant is one of the most importantleguminous forage crops with high nutritional value and higherproduction continuing for many seasons and can be sown in spring or fall, and does best on well drained soils with a neutral levels of pH 6.8 – 7.5, alfalfa requires sustained levels of potassium and phosphorus to grow well, it is moderately sensitive to salt levels in both the soil and irrigation water [5].pH is one of important environmental factors which effect on the rate of effective survival rhizobia, the changing of pH level means adding or subtracting hydrogen ions, changes in pH have the most noticeable effects on bacterial enzymes, extreme changes in the pH balance of the local environment for bacteria tend to kill them [6].ThepH affect the melting of some nutrient elements in the soil, as well as the rate of absorption of some elements, and inhibits the growth of some crops like alfalfa [7]. Fe is the most important element which needs in the formation of the compound leghemoglobin which plays an important role in the transfer of oxygen into the root node, the presence of oxygen in the root nodules would reduce the activity of the oxygen sensitive nitrogenase which responsible for the fixation of atmospheric nitrogen [8]. Due to the less studies in this field in Iraq, this paper aimed to isolate *E. meliloti*from different locations of the Babylon province from roots nodules of alfalfa plants and focused on the effects of pH on symbiotic relationships between these parteners .

II. Materials And Methods

2.1Bacterial isolates

After collection of alfalfa plants from 10 different areas of Babylon province, mature pink nodules on root hairs were cut and surface sterilized using 95% of ethanol for 1 minute and washed in sterile distilled water for 5 times then the nodules were crashed with the help of glass rod in 1 ml of physiological saline solution.The suspension was spreadedon TY media and incubated at 28 °C for 48 hours.The bacteria were identified depending on traditional methods as described by [9] , and PCR technique [10].

2.2 Plant cultivar

Seeds of alfalfa (*M. sativa*L.) plants were purchased from local markets of Hillacity,Iraqand theirplantsclassified in the lushly of Babylon University [9].

2.3Media for *E. melilot*iisolates

Trypton yeast extract (TY) medium used for growing of isolates andprepared according to [11].TY swarm plates is the same as TY medium except for reduction of agar concentration to 0.3% (W/V). This medium was used to test the motility and ability of *E. melilot*iisolates to produce β-(1,2)-glugans. Mannitolsalt yeast extract medium (MSY) was used to test the ability of *E. melilot*iisolates to produce β-(1,3)-glugans, cellulose fibrils and lipopolysaccharides (LPS)[11].

2.4Media for alfalfa (*M. sativa* L.) plants

Water agar medium contained1%(W/V) agar was used for germination of alfalfa seeds(Vincent,1970) and nitrogen free plant growth medium was used for growing of alfalfa plant [12].

2.5Determination of cell surface carbohydrate molecules

To determin the cyclic β-(1,2)-glucans,the isolates were spotted on TY swarm plates[13].β-(1,3)-glucans were determined by streaking the bacterial isolates on MSY agar plates containing 0.02% (W/V) of aniline blue [14].Cellulose fibrils were determined by streaking the isolates on MSY agar plates containing 0.02% (W/V) of congo red [15].Sodium deoxycholate was added to MSY medium atthe rateof 1 mg/ml before autoclaving to test the ability of isolates to produce LPS[16].All plats were incubated at 28°C for 2 hours.

2.6Molecular identification

Whole DNA was extracted from pure culture isolates using DNA wizard genomic extraction kit (Promega, Madison, WI, USA).The materials and kit used for PCR technique are listed in Table 2.

Table 2. Materials and Kit used for PCR technique

Material	Describe
DNA Marker 100-1000bp	DNA ladder used to determine the size of double strand DNA fragments and consists of 10 pieces (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp).
Go tag Green Master Mix, 2X	Cotain Go Tag DNA Polymerase , 2X Go Tag Reaction Buffer (pH 8.5) , MgCl2 , dATP , dTTP , dCTP , dGTP .
Wizard Genomic Extraction kit	Used to extracte DNA from bacterial isolates and consists of cell lysis solution , nuclei lysis solution , protein precipitation solution, RNase , Tris EDTA

The primers of PCR were synthesized by Bioneer company- Korea as described by [10].Nodbox1, nodbox3, mucRf, and mucRr were recognized homologous conserved regions in the *E. melilot*igenome. The conditions of PCR amplification of genomic DNA of *E. melilot*iwere 95°C for 30 S, 53°C for 45 S, and 72°C for 30 S for 25 cycles, followed by 7-min elongation step at 72°C. Each primer was added at a concentration of 0.4 µM. (Table 3.)

Table 3.PCR primers and conditions used in this study. (Bioneer company- Korea)

Primer	Sequence (5 → 3)	PCR Amplicon (bp)	Denaturaion, AnnelingandExtension	No. of cycle	Source
nodbox1	TCTTTTCTTATCCATAGGGTGG	646	95°C / 30 S 53°C / 45 S 72°C / 30 S	25	Sanchez-Contrerasetal., (2000)
nodbox3	ACGGATCGTCCTCGAAG				
mucRf	ATGACAGAGACTTCGCTCGGT	431			
mucRr	TCACTTGCCGCGACGCTT				

2.7Preparation of reaction mixture

The reaction mixture was prepared according to the instructions of the manufacturer (Promega USA). The total volume of reaction was 50 µL consist of 12.5 µL from 2X Go Taq Green Master Mix, 5 µL from each primer , 5 µL from DNA template , then the volume complete to 50 µL by free nuclease water. Negative control contains all contents without DNA template was also used.

2.8Agarose gel electrophoresis

The electrophoresis was performed according to[17].The PCR products were run on horizontal agarose gel (1%) stained with ethidium bromide, for 90 min. and 70 volt. The DNA bands were photographed by Gel documentation system (Biometra Co. Germany)

2.9Preparation of seedings

Normal and undamaged seeds of alfalfa plants were selected,washedwith distilled water for 10 minutes, surface sterilized with 70% of ethanol alcohol for one minute then washed with distilled water followed by treatment with sodium hypochlorite (NaOCl) for 15 minutes after that washedwith distilled water 3 times, spread on 1.0 % (w/v) water agar medium and incubated inverted at 25°C for two days in dark place [9].

2.10Plant tests and growth conditions

Three healthy grown seedlings with two days old transferred to the slants of nitrogen free plant growth medium with different pH (pH6.5, pH7, pH7.5, pH8 and pH8.5) in 30 X 2.5 cm glass tube containing 25 ml of this medium [12].The lower portion of each tube was wrapped with black paper to prevent the root system from exposure to light. These tubes were incubated in plant growth chamber (Memmert, Germany) under conditions 25°C , 70-80% humidity and 2000 lux of visible light for six weeks. 16 hours light and 8 hours dark cycle were maintained. 0.5 ml of *E. melilotii*solate cells suspension was dispensed into each tube having three days old seedlings. Control tubes containing three seedlings each received 0.5 ml of physiological saline solution. Three tubes containing three seedlings each were used for each tretment. Many parameters characteristic of each plant were recorded such as time of appearance of first nodule , nodule number, nodule shape, nodule colours, shoot length and shoot dry weight.Tomeasure the latter, plant shoots were collected, dried in an oven at 60 C° for three daysand then weighted. Nitrogen fixation was indirectly evaluated by scoring the plants Fix^+ or Fix^- on the basis of nodule colour and/or plant matter production [2].

2.11Alfalfa root hair deformations

The root hairs of 4-10 days old plants after inoculation with isolates were removed, washed with sterile water and cut into 1 cm long pieces . The root pieces were stained with methylene blue (0.01 % W/V) for 15 minutes, washed with sterile water, place on clean glass slide and covered with cover slip and then examined at 10X and 40X magnifications under light microscope for observing root hair deformations (curling , waviness ,bulging and swelling , shepherd's crook with hyaline spot , formation of infection thread and formation of nodules [18].

2.12pH optimization

Different concentrations of hydrogen ion (pH5 , pH6 , pH7 , pH8 , pH9 and pH10) in Ty medium were used.Each plate inoculated with 0.1 ml of bacterial isolates(approximately contain 3×10^5CFU/ml)using pour plating techniqueandincubatedat 28 °C for 48 hours[19].

2.13Statistical analysis

For analysis the data that collection from this study , one way ANOVA test which described by [20].was used for this purpose, all values were expressed as mean ±standard error of mean.

III. Results And Discussion

3.1 Isolation of *E. melilotii*solates

A total of ten local isolates of E.melilotiwere isolated from root nodules of M. sativiaL. from different areas of Babylon province. These isolates were chosen for the further experimsents.Thenumber, name and place of isolates are listed in Table1.

Table 1 .Number , name and place of bacterial isolates .

No. of Isolate	Name of Isolate	Place of Isolates
1	Em1	Hilla-AljazaeerQuartar
2	Em2	Hilla-Babylon university
3	Em3	Babylon-Nile area
4	Em4	Babylon-Abo garaq area
5	Em5	Babylon-Almethateya area
6	Em6	Babylon-Almahaweel area
7	Em7	Babylon-Alkefel area
8	Em8	Babylon-Alqasim area
9	Em9	Babylon-Indian dam area
10	Em10	Hilla-Alwardia area

3.2Identification of *E. melilotii*solates

The isolates were identified depending on morphological properties of the cells and colonies of these isolates, and thecell surface carbohydrate molecules (Fig. 1.).

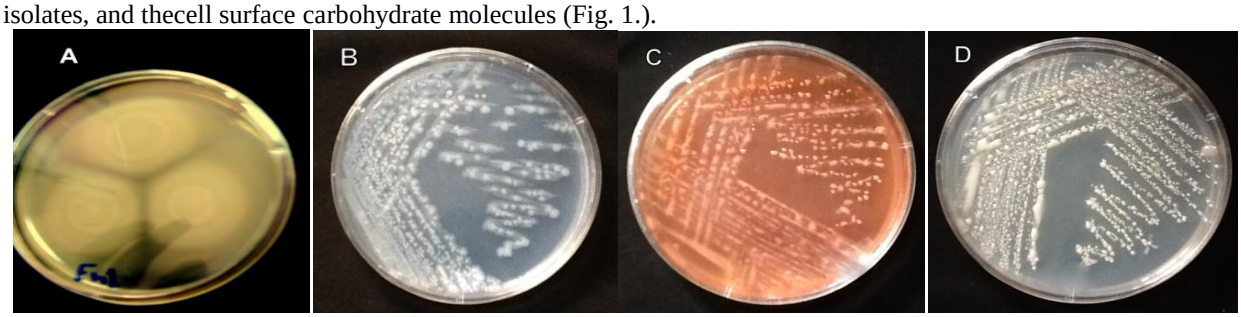


Figure1 .Production of cell surface carbohydrate molecules of E.meliloti isolates.

A. Cyclic β-(1,2)-glucansproduction showed swarming on swarm plates. **B**. β-(1,3)-glucansproductionwhichdid not show binding with aniline blue dye on MSY medium.**C**. Cellulose fibrilsproductionshowed red colonies due to took up congo red dye on MSY medium.**D.** LPSproductionshowed growth on MSY medium containing sodium deoxycholate.

All bacterial isolates showed swarming on swarm plates therefore, these isolates were motile and had ability to produce of cyclic β-(1,2)-glucans. The isolates did not uptake aniline bluedye and unable to produce of β-(1,3)-glucans. Rhizobial cell surface is a complex conglomerate of various polysaccharides that play a major role in the development of symbiotic nodules. [21].found that only *E. melilotii*was unable to produce β-(1,3)-glucans but all rhizobia can produce β-(1,2)-glucans. The absence of cyclic glucan synthesis affect the invasion capacity of the bacteria,mutantsdefective in the synthesis of this polysaccharide induce empty nodules and did not induce the formation of the infection threads [13] [22].The isolates were uptake congo red dye and showed red colonies due to produce of cellulose fibrils.[23] , showedthatcongo red absorption has been used as amarkerfor identification of rhizobia.The isolates were able to utilized sodium deoxycholate and produced lipopolysaccharide .[24].found that mutation in production of LPS of E. meliloticaneffect its ability to utilize sodium deoxycholate. [25]reported that the O-antigen structure was changed when rhizobia subjected to seed or root compounds. During the differentiation of the bacterium into bacteroid, LPS undergoes changes in its structure [26].A general observation is that the LPS changes during symbiosis lead to an increasing of surface hydrophobicity that influence the interaction between bacterial and plant cell membranes and could be relevant for the sincronic division bacteoid / symbiosome[27] [28]. The molecular identification of E. melilotiusingPCR technique (Fig. 2) showedtwo regions of the *E. melilotii*genome: the nodbox 4 promoter and the mucR gene. Both of these regions are specific for *E. melilotii*and are highly conserved and ubiquitous in the species[29].Furthermore, they are located in different replicons; the nodbox 4 region is located in one of the symbiotic megaplasmids[30] , and mucR is located in the chromosome [31].Simultaneous detection of both regions should increase the specificity of the method compared to methods based on use of a single primer pair. The nodbox 4 region was amplified using primers nodbox1 and nodbox3. [10]found that PCR amplification of the nodbox andmucRloci is a reliable and rapid method for identification of *E. melilotii*strains (when primers nodbox1, nodbox3, mucRf, and mucRr were used simultaneously the product was two discrete bands at 646 and 431 bp were amplified from all of the *E. melilotii*strains), especially when high numbers of other bacteria are expected to be presented in nodules. Therefore, this technique can be used to generate a strain collection from field samples with reduced laboratory effort in a short period of time. Furthermore, the two amplicons used in this study added new probes to those already available for identification of E. meliloti. Optimization of this PCR procedure allowed simultaneous detection of the two loci with a single PCR

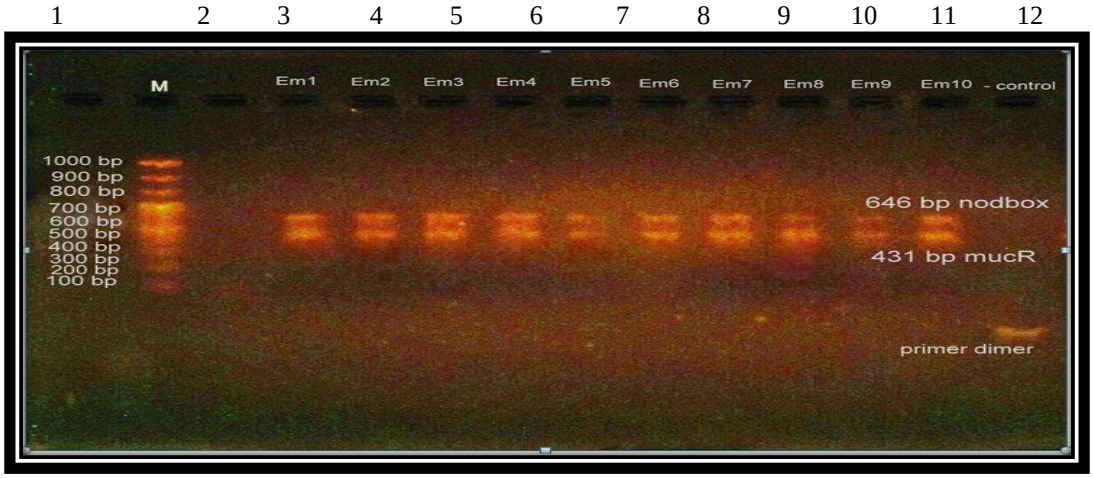


Figure 2 .Agarosegelectrophoresis of the amplified products of *E. melilotii*solates on (1%) agarose gel for 90 min. and 70 V. Lane1 : ladder 1 Kb .Lanes 2 to 11 : Em1 to Em10, respectively.Lane 12 : negative control.

3.3 Infection phenotypes on alfalfa root hairs and formation of nodules

When the methylene blue stained root portions of alfalfa plant inoculated with isolates, observed under light microscope, it was found that all isolates werenormal in elicitation of root hair deformations, shepherd´s crook with hyaline spote, infection thread and formation of nodules. However, the root hairs of uninoculated controls were straight and devoid of the infection phenotypes (Plats 1 and 2).The symbiotic interaction starts when the bacteria colonize the root surface and induce curling of the root hair tips,this is followed by cell wall invagination and the formation of an infection thread that grows within the root hair[32] [33].

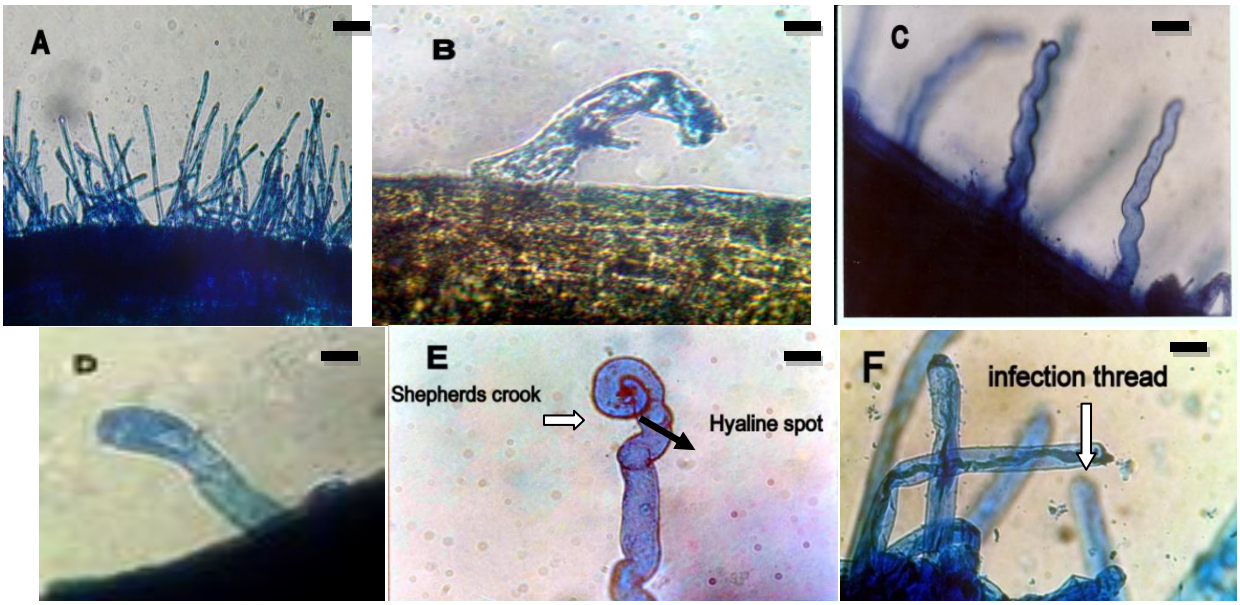


Plate 1 . Light microscopic observations of root hairs of *M. sativa* L. after 4- 10 days of inoculated with E. melilotiisolates . A. Uninoculated control showing intact, straight root hairs. B. Curling root hair. C. Waviness root hair. D. Bulging or swelling root hair. E. Formation of shepherd,s crook showing the 360° curvature of the root hair tip with hyaline spot F. Infection thread formation which elongation at the base of the root hair cell. Bars: A, 100 µm (X100); B,C,D,Eand F, 25µm (X400).

The infection thread traverses the outer cell layers to reach the nodule primordium, which isinitiated by the reactivation of differentiated cells of the root cortex for division. Within the infection thread the rhizobia multiply but remain confined by the plant cell wall. As the primordium develops to a nodule, bacteria are released from the tip of the infection thread by endocytosis and differentiate into bacteroids surrounded by the peribacteroid membrane, this process will continue to form a mature nodules. The symbiotic interaction involves an exchange of complex molecular signals that confer specificity. Legume roots and seeds exude different substances: sugars, amino acids, dicarboxilic acids and various aromatic compounds such as some

flavonoids in mixtures that differ between species [34].Rhizobia respond to these compounds because they have one or more nodD genes, which encode regulator proteins that activate the other nod genes when they interact with appropriate plant signal compounds. Once activated, the nod genes direct the synthesis of Nod factors (NF), a family of lipochitin oligomers (LCO),which acting as morphogens, initiating the nodulation program of the host plant [35] [36].The induction of nitrogen-fixing root nodules on leguminous plants by rhizobia requires multiple interactions between the two partners. In different Rhizobium and Bradyrhizobium species, several gene sets (nod genes) have been identified which control the early steps of nodulation [37].The common nodulation genes (nodABC) are essential and conserved in all rhizobia, both functionally and at the DNA sequence level[38].Other sets of genes determine the host-specificity of nodulation (hsnABCD genes) [39].

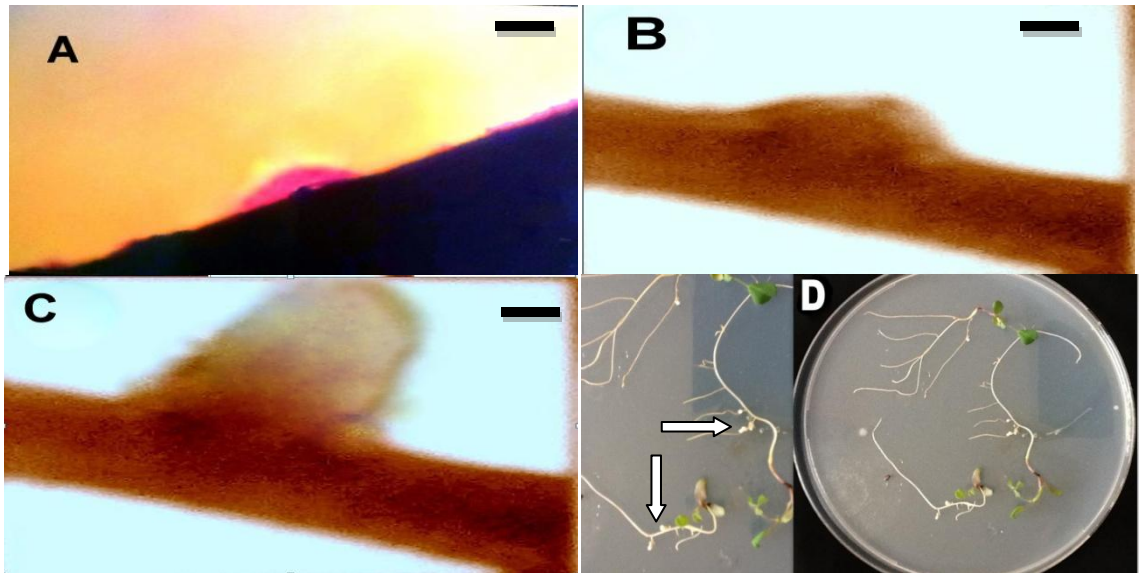
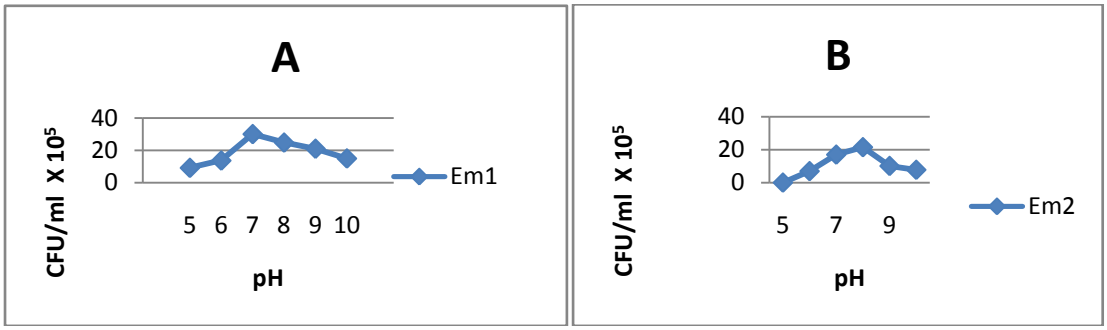


Plate 2 . Light microscopic observations of root hairs of *M. sativa* L. after 2-4 weeks of inoculated with E. meliloti. A, B and C. Intiation nodule structure.D.formation of nodules.Bars: A,B and C, 25µm (X400); The arrows in E, indicate the positions of the nodules.

3.4 Effect of pH on growth of *E. meliloti*isolates

The bacterial isolates have a range of pH 6-10 except for Em1 and Em8 isolates have ability to growth on pH5, the pH of isolates Em1, Em3, Em4, Em6 and Em8 was pH7 and for remaining isolates was pH8 (Fig.3).[40] showedthatacid pH limits the persistence of Rhizobium strains in soil, and the nodulation and nitrogen fixation of legumes, they tested the ability of 45 Rhizohirrm, Azorhizohirrm and Bradyrhizobium strains to produce colonies on agar medium at pH 4to pH 7,only R. tropici UMRI 899 grew at pH 4in unbuffered medium, though 6 strains of R. tropici and 3 Bradyrhizobium strains grew at pH 4.25, and 15 strains grew at pH 4.5.



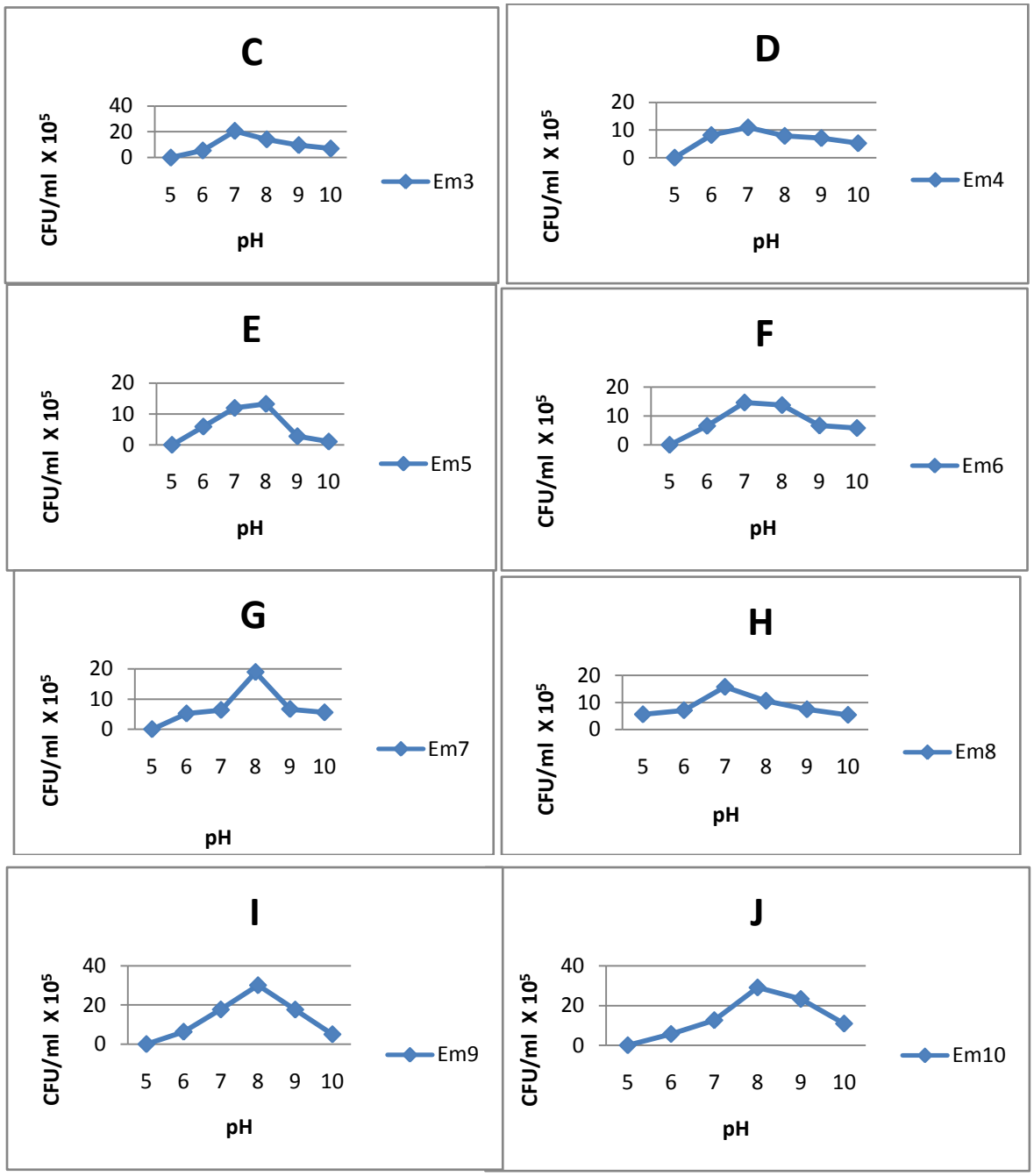


Figure 3 . Effect of pH on growth of *E. melilotii*solates incubated at 28°c for 48 hours.

3.5Characteristics of alfalfa plants nodulated by *E. melilotii*solates at different pH.

The nodule and shoot characteristics of alfalfa plants nodulated by the *E. melilotii*solates are given in Table 5,6,7,8 and 9. The data presented in these Tables are being listed only after reisolation of the particular isolate from nodules and checking for their markers (Koch´s postulate).Em2, Em3, Em4 , Em5 , Em6 , Em7 , Em9 and Em10 isolates at pH 6.5 and all isolates at pH 8.5 did not form nodules on the roots of alfalfa plants. The isolates Em1 and Em8 at pH 6.5; Em2, Em3 , Em4 , Em5, Em6, Em7 and Em10 isolates at pH 7; Em3, Em9 and Em10 isolates at pH 7.5 and Em1, Em3, Em5, Em6, Em7, Em8 and Em9 isolates at pH 8formed small and white (suggesting a lack of leghaemoglobin) nodules on the roots of alfalfa plants that were symbiotically ineffective.The remaining isolates elicited normal, cylindrical and pink (indicating the presence of leghaemoglobin) nodules on roots of alfalfa plants that were symbiotically fully effective. The mean height and dry shoot weight of plants nodulatedby Em1, Em5, Em6, Em8 and Em9 isolates at pH 7; Em1-Em10isolates at pH 7.5 and Em2, Em3, Em4 and Em10 isolates at pH 8 differed significantly with the uninoculated controls ($P<0.05$).These plants appeared healthy and green (indicating that nitrogen was being fixed).The mean time

taken for the appearance of first nodule after inoculation with the isolates ranged from 8 days for Em2 isolate at pH 7.5 to 21.6 days for Em8 isolate at pH 6.5; mean number of nodules per plant varied from 0.6for Em8 isolate at pH 6.5 to 11.3 for Em6 isolate at pH 7; mean shoot length per plant differed from 2.3cmforEm3 isolate at pH 6.5 to 18.5 cm for Em8 isolate at pH 7.5, whereas 8.3 cm for the uninoculated controls at pH 7.5; mean dry shoot weight per plant varied from 3mgforEm4 isolate at pH 6.5 to 18.6mgforEm6 isolate at pH 7.5, whereas 7.6 mg for the uninoculated controls at pH 7.5. [41]showed soil acidification is one of the environmental factors that more strongly hampers the establishment of an effective symbiotic interaction between rhizobia and leguminous plants. *E. meliloti*and the acid-tolerant Rhizobium sp. strain LPU83 are able to nodulate alfalfa plants at pH 5.6 but both exhibit a delayed nodulation and a reduction in the number of elicited nodules. [42]found that collection of 465 isolates of rhizobia were characterized for acid tolerance revealed the existence of 15 acid-tolerant isolates which were able to grow at pH 5.0 and formed nodules in alfalfa with a low rate of nitrogen fixation.[43]reported that biological nitrogen fixation is a phenomenon occurring in all known ecosystems and thesymbiotic nitrogen fixation is dependent on the host plant genotype, the rhizobia strains and the interaction of these symbionts with the pedoclimatic factors and the environmental conditions.However,extremes of pH affect nodulation due to reducing the colonization of soil and the legume rhizospher. Highly alkaline soils (pH > 8) tend to be high in sodium chloride, bicarbonate, and borate, and are often associated with high salinity which reduce nitrogen fixation and highly acidic soils (pH < 4) frequently have low levels of phosphorus, calcium, and molybdenum and high concentrations of aluminium and manganese which are often toxic for both partners; nodulation is more affected than host-plant growth and nitrogen fixation.

Table 5. Effect of pH6.5 on the symbiotic relationship between alfalfa plants and bacterial isolate

Bacteral isolate	Nodule Characteristics				Shoot Characteristics	
	Time of first nodule appearance in days (mean ± SE)	Nodule/plant (mean ± SE)	Shape	Colour	Shoot length cm/plant (mean ± SE)	Dry shoot weight mg/plant (mean ± SE)
Control (no inoculum)	Nil	Nil	Nil	Nil	3.6 ± 0.3	4.3 ± 0.8
Em1	20.3 ± 1.4	1 ± 0.5	Irregular	White	4.3 ± 0.6	4.6 ± 0.3
Em2	Nil	Nil	Nil	Nil	3 ± 1.1	4.6 ± 0.6
Em3	Nil	Nil	Nil	Nil	2.3 ± 0.3	3.6 ± 0.3
Em4	Nil	Nil	Nil	Nil	3.3 ± 0.6	3 ± 0.5
Em5	Nil	Nil	Nil	Nil	3 ± 0.5	3.6 ± 0.3
Em6	Nil	Nil	Nil	Nil	3.6 ± 0.4	4.6 ± 0.6
Em7	Nil	Nil	Nil	Nil	3.3 ± 0.8	4.6 ± 0.3
Em8	21.6 ± 1.1	0.6 ± 0.3	Irregular	White	4 ± 0.5	4.3 ± 0.3
Em9	Nil	Nil	Nil	Nil	2.6 ± 0.6	3.3 ± 0.3
Em10	Nil	Nil	Nil	Nil	3.6 ± 0.8	4.3 ± 0.8

Each value is a mean of three plants ± standard error .

Table 6. Effect of pH7 on the symbiotic relationship between alfalfa plant and bacterial isolates .

Bacteral isolate	Nodule characteristics				Shoot characteristics	
	Time of first nodule appearancein days (mean ± SE)	Nodule/plant (mean ± SE)	Shape	Colour	Shoot length cm/plant (mean ± SE)	Dry shoot weight mg/plant (mean ± SE)
Control (no inoculum)	Nil	Nil	Nil	Nil	3.3 ± 0.8	5.3 ± 0.8
Em1	15 ± 1.5	4.3 ± 1.2	Cylindrical	Pink	7.6 ± 0.8*	10.6 ± 0.8*
Em2	15.3 ± 1.2	1.3 ± 0.3	Irregular	White	3 ± 0.5	3.3 ± 0.8
Em3	15.6 ± 0.8	3.3 ± 0.6	Irregular	White	4.6 ± 0.8	6.6 ± 1.2
Em4	16 ± 1.1	3.6 ± 0.8	Irregular	White	5.3 ± 1.3	7 ± 1

Em5	16.6±0.8	2.3±0.8	Irregular	White	7±1.5*	9.3±1.2*
Em6	15.3±0.6	3±0.5	Irregular	White	8.6±0.8*	10.6±1.4*
Em7	17.3±0.3	1.6±0.3	Irregular	White	4±0.5	5±0.5
Em8	16.6±1.4	4±1.5	Cylindrical	pink	9±1.1*	7±1.1*
Em9	15.3±0.8	2.6±0.8	Cylindrical	pink	10.6±1*	10.3±1.2*
Em10	14.6±1.3	1.6±0.6	Irregular	White	3.6±0.6	4.3±0.4

Each value is a mean of three plants ± standard error .* Significant difference with control ($p<0.05$).

Table 7. Effect of pH7.5 on the symbiotic relationship between alfalfa plant and bacterial isolates .

Bacteral isolate	**Nodule characteristics**				**Shoot characteristics**	
	Time of first nodule appearance in days (mean ± SE)	**Nodule/plant (mean ± SE)**	**Shape**	**Colour**	**Shoot length cm/plant (mean ± SE)**	**Dryshoot weight mg/plant (mean ± SE)**
Control (no inoculum)	Nil	Nil	Nil	Nil	8.3±0.3	7.6±0.3
Em1	9.6±0.8	8.6±1.2	Cylindrical	Pink	11.3±1.2*	12.3±0.6*
Em2	8±0.5	10.3±1.4	Cylindrical	pink	15±1.5*	15.6±1.8*
Em3	8.6±0.3	9±1	Irregular	White	14.6±1.3*	16.6±0.6*
Em4	10.3±0.8	7±0.5	Cylindrical	pink	17.3±1.2*	17.6±1.2*
Em5	10.6±0.8	8.3±0.3	Cylindrical	pink	17.3±0.3*	18.3±1.6*
Em6	9.3±0.8	11.3±0.8	Cylindrical	pink	17.6±1.3*	18.6±0.8*
Em7	9.3±0.3	8.6±1.2	Cylindrical	pink	15.6±0.3*	16.6±1.2*
Em8	8.6±0.8	7.6±1.3	Cylindrical	pink	18±0.5*	19±0.8*
Em9	8.3±0.3	7.3±0.8	Irregular	White	15.3±0.5*	10±0.5*
Em10	10±0.8	9±0.5	Cylindrical	White	12.6±1.2*	14.3±0.8*

Each value is a mean of three plants ± standard error .* Significant difference with control($p<0.05$).

Table 8.Effect of PH8 on the symbiotic relationship between alfalfa plant and bacterial isolates.

Bacteral isolate	**Nodule characteristics**				**Shoot characteristics**	
	Time of first nodule appearance in days (mean ± SE)	**Nodule/plant (mean ± SE)**	**Shape**	**Colour**	**Shoot length cm/plant (mean ± SE)**	**Dry shoot weight mg/plant (mean ± SE)**
Control (no inoculum)	Nil	Nil	Nil	Nil	6.3±0.8	8.3±0.3
Em1	9.3±0.1	4.6±0.6	Cylindrical	White	7.6±0.3	10±1.1
Em2	10±0.4	6±0.5	Cylindrical	Pink	10.3±0.8*	11.3±0.6*
Em3	9.3±0.7	6±1	Irregular	White	9.6±1.2*	11.6±0.8*
Em4	9.6±0.9	5±1.1	Cylindrical	Pink	10.6±0.8*	11.3±1.2*
Em5	9.3±0.7	4.3±0.3	Irregular	White	8.3±0.3	8.6±0.3
Em6	10±0.9	4.6±0.8	Cylindrical	White	7±1.1	9.3±0.8
Em7	9.6±0.9	4.6±0.3	Cylindrical	White	7.6±0.8	9±0.5
Em8	10.3±0.9	4±1.1	Cylindrical	White	7.6±0.3	8.3±0.3
Em9	11.6±0.7	5±0.5	Irregular	White	8.6±0.6	10±0.5
Em10	11±0.4	7.3±0.3	Cylindrical	Pink	13.6±0.3*	15±0.5*

Each value is a mean of three plants $\pm$ standard error .* Significant difference with control($p<0.05$).

Table 9. Effect of PH8.5 on the symbiotic relationship between alfalfa plant and bacterial isolates .

Bacteral isolate	Nodule characteristics				Shoot characteristics	
	Time of first nodule appearance in days (mean $\pm$ SE)	Nodule/plant (mean $\pm$ SE)	Shape	Colour	Shoot length cm/plant (mean $\pm$ SE)	Dry shoot weight mg/plant (mean $\pm$ SE)
Control (no inoculums)	Nil	Nil	Nil	Nil	5.6 ± 0.3	6 ± 0.8
Em1	Nil	Nil	Nil	Nil	3 ± 0.5	4 ± 0.5
Em2	Nil	Nil	Nil	Nil	4.3 ± 0.8	4.6 ± 0.8
Em3	Nil	Nil	Nil	Nil	5 ± 0.5	5.6 ± 0.6
Em4	Nil	Nil	Nil	Nil	6 ± 1	6.6 ± 0.8
Em5	Nil	Nil	Nil	Nil	5.6 ± 1.2	6 ± 0.5
Em6	Nil	Nil	Nil	Nil	4.3 ± 0.8	5 ± 0.5
Em7	Nil	Nil	Nil	Nil	5.3 ± 0.3	6.3 ± 0.3
Em8	Nil	Nil	Nil	Nil	4.6 ± 0.3	5 ± 0.5
Em9	Nil	Nil	Nil	Nil	5.3 ± 1.2	6 ± 1.1
Em10	Nil	Nil	Nil	Nil	5.6 ± 1.2	6 ± 0.5

Each value is a mean of three plants $\pm$ standard error .

IV. Conclusion

It is concluded that all bacterial isolates in this study were neutrophilesor little alkaliphilesand did not show any acidophilic isolates, because most of the Iraq soil in the center and south of Iraqwere alkaline due to the high rate of insoluble ions. However, still there are many gaps in our knowledge about nitrogen fixing bacteria in Iraq which needs farther investigations.

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