# THE SPONTANEOUS NODULATION OF ALFALFA GROWN IN SOIL POLLUTED WITH PETROLEUM OIL

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Pot experiment was conducted under green house conditions to study the phytoremediation activity of alfalfa, *Mediacago sativa* in soil polluted with crude petroleul oil at rates of (4%, 8% and 10%). Results showed enhancing of plant growth at 4% and 8%, while at 10%, no growth was observed. The appearance of oranges-like nodules on plant roots at 8% concentration after 42 days from planting date was observed. Nodules were large in size and few in number, some of them were oval and the others elongated.

Both nodules, shapes contain cells of bacteria and by using Biology Technique for identification process, these isolates were identified as *Ochrobactrum* (oval nodules) and *Cellulosimicrobium* (elongated nodules).

Both genera were recorded for the first time in Egypt during this study. To be sure that the previous isolated have this phenomenon, seeds of selected plants were inoculated with each of them and cultivated in sterile sandy soil under the same rate of crude oil (8%).

Formation of nodules were observed also after 42 days after planting date. In vitro, efficiency of the two isolates for degrading petroleum oil was investigated. It was observed that the residue of petroleum oil by *Ochrobactrum* was 28.2%, while the residue of petroleum oil by *Cellulosimicrobium* reached 21.9%.

**Keywords:** *Medicago sativa*, Alfalfa, nodulation, petroleum hydrocarbons *Ochrobactrum* and *Cellulosimicrobium*.

Various plants have been identified for their potential to facilitate the phytoremediation of sites contaminated with petroleum crude oil. In the majority of studies, grasses and legumes have been tested for their potential, in this regard. Legumes are thought to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen, i.e. legumes do not have to compete with microorganisms and

other plants for limited supplies of available soil nitrogen at oil contaminated sites.

Interaction between leguminous plants and the soil bacteria rhizobia results in the development of the root nodules. In the last few years, there has been an increasing number of researches focused on nodulation in the absence of *Rhizobium* and spontaneous nodulation or nodulation by organisms other than *Rhizobium*. Most bacteria that establish a symbiosis with legume plants, including some non rhizobial species of *Methylobacterium* (Sy *et al.*, 2001) and *Devosia* (Rivas *et al.*, 2003) belonging to the α subclass of *Proteobacteria*, although some species from genera of the β subclass such as *Ralstonia* and *Burkholderia*, can also nodulate legumes (Chen *et al.*, 2001; Moulin *et al.*, 2001 and Vandamme *et al.*, 2003).

The aim of this research is to study the effect of different levels of petroleum crude oil on alfalfa nodulation during the remediation process.

## MATERIALS AND METHODS

**Cultivar**

Alfalfa seeds (*Medicago sativa*) was provided by the Agriculture Research Center, Giza, Egypt.

**Soil used**

Sandy soil was collected from 10$^{th}$ of Ramadan area (total nitrogen 0.04%, organic carbon 0.4%, EC 2.5 dS/m, $CaCO_3$, 2% and pH 7.2).

**Crude oil characters**

The crude oil used was behaved from Balayim, general characterization of 28.84 API MARINE BALAYIM. Crude oil was detected in Egyptian Petroleum Research Institute (Table 1).

**Treatments**

Plants under investigation were grown in sandy soil using earthen pots and were filled with 10 kg/pot unsterilized soil and pots were divided into groups and treated as follows:

1-Control (without adding crude oil)

2-Soil treated with crude oil at different levels, 4%, 8% and 10%.

Fertilization rates were 60 kg N/fed. (ammonium nitrate 33.5%), 25 kg $P_2O_5$/fed. (Ca-superphosphate 15.5%) and 48 kg $K_2O$/fed. (potassium sulphate 48%).

**Nodule Formation of Alfalfa Roots**

After 6 weeks from planting under different levels of crude oil, spontaneous nodules were observed on roots in a small percentage and at two types, oval and elongated shapes.

**Isolation of Bacteria from Plant Nodules**

Bacterial strains were isolated from root nodules of *Medicago sativa* growing in pot experiment under green house condition in the presence of crude oil. Isolation was made according to the method of Vincent (1970) with Tryptic Soy Agar (TSA).

**Table (1). General Characteristics of Balayim Crude Oil.**

| Character | | Method | Results |
|---|---|---|---|
| **Specific gravity @ 60/60 °F** | | ASTM D 1298 | 0.8825 |
| **°API gravity** | | ASTM D 1298 | 28.48 |
| **Sulphur content** | **Wt %** | ASTM D 4294 | 2.10 |
| **Ash content** | **Wt %** | ASTM D 482 | 0.021 |
| **Salt content** | **PTB** | ASTM D 3230 | 38.0 |
| **Carbon conradson residue** | **Wt %** | ASTM D 189 | 7.5 |
| **Water content** | **Vol %** | ASTM D 95 | 0.05 |
| **Sediment content** | | ASTM D 437 | 0.014 |
| **Viscosity RED 1 @ 100 °F** | **Sec** | IP 70 | 85 |
| **Viscosity Kinematic @ 37.8 °C** | **cSt** | ASTM D 445 | 19.93 |
| **Viscosity Kinematic @ 50 °C** | **cSt** | ASTM D 445 | 13.59 |
| **Asphaltene content** | **Wt%** | IP 143 | 3.9 |
| **Wax content** | **Wt%** | UOP 46 | 4.2 |
| **Pour point** | **°F** | ASTM D 97 | 40 |
| **Vapour pressure RED @ 100 °F** | **kg/Cm²** | ASTM D 323 | 0.4 |
| **Hydrogen sulphide** | **PPM** | MOBIL | 3 |
| **Mercaptan sulphur** | **PPM** | UOP 163 | 10 |
| **Inorganic acidity** | **mg KOH/g** | IP 182 | NIL |
| **Total acidity** | **mg KOH/g** | ASTM D 664 | 0.11 |
| **Nitrogen content** | **Wt %** | UOP 384 | 0.23 |
| **Metals : PPM , WT** | | | |
| **Vanadium content** | **PPM** | I P 285 | 45 |
| **Nickel content** | **PPM** | | 40 |
| **Light hydrocarbons** | | IP 320 | |
| **Total C4 & Lighter** | | | 1.302 |
| **Ethane** | | | 0.002 |
| **Propane** | | | 0.4 |
| **Iso- Butane** | | | 0.3 |
| **N – Butane** | | | 0.6 |

Wt% :Weight % PTB : Pound /1000 barrel Vol. :Volume Sec : Second
cSt :Centistock kg /cm² :Kilogram/centimeter square
ASTMD :American Standard Testing Method IP :Institute of Petroleum
API :American Petroleum Institute UOP :United Oil Petroleum

**Nodulation tests**

To be sure that nodules which formed on alfalfa roots were due to the inoculation by the obtained two isolates, surface-sterilized seeds of alfalfa that were planted in sterile sandy soil. Then ten plants were inoculated with 10 ml of bacterial suspension containing $7\times10^8$ cells/ml. The cultures used to inoculate alfalfa plants were purified "from a single colony" after 24 hrs of incubation at 30°C and cultivated on TSA medium.

The inoculated plants were placed for 7 weeks in a greenhouse, as a negative control uninoculated *Medicago sativa* plants were used. After 6 weeks, the nodules were observed again.

**Anatomical Study of Nodules**

Specimens were killed and fixed for at least 48 hrs in formalin-acetic acid- alcohol (F.A.A.). The selected material were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcoholic series, embeded in paraffin wax of melting point 56°C. Sectioned with crystal violet-erythrosin, cleared in xylene and mounted in canada balsam.

**Identification of Bacterial Isolates**

The microbial isolates have been identified by using the BIOLOG technique at the department of Epidemiology and Etiology which concerns to the U.S. Naval Medical Research Unit No. 3 / Cairo, Egypt.

**Determination of Oil Degradation**

The two isolates were tested for their biodegradation capacity of crude petroleum oil. One milliliter of bacterials suspension ($10^6$ cells) were introduced into 250 ml conical flasks containing 50 ml of synthetic medium supplemented with sterilized petroleum oil 0.5% (w/v). Six flasks were used for each organism, three for the evaluation of the biodegradation capacity of the organism, and the other three flasks each received 0.2% formaldehyde to evaluate the loss of crude oil due to evaporation and non biological factors. All flasks were incubated at 30°C on rotary shaker operated at 100 rpm for 21 days.

At the end of the incubation period, the remaining oil in each culture was recovered using the method described by Oudot (1984). The oil residue was weighed, suspended in n-hexane and filtered to remove the insoluble fraction (asphaltene).

## RESULTS

**Nodulation**

Oval and elongated nodules formed on the main root of afalfa after 42 days after sowing are shown in Fig (1).

**Description of Bacterial Strains Isolated from Nodules**

**1-Strain isolated from an oval nodule**

Cells are non motile, gram negative rods, non spore-forming. Good growth was observed on Tryptic soy agar, yeast extract mannitol broth, MacConkey agar, nutrient agar and blood agar at 30 -35°C and able to grow in the presence of 5% NaCl. Carbon source utilization includes glucose, lactose and sucrose. The revision and the comparison results with American Type Culture Collection ATCC has been done and the strain was identified as *Ochrobactrum anthropi* strain number BAA -749.

**2-Strain isolated from an elongated nodule**

Cells are non motile, gram positive rods, non spore-forming and exhibited good growth on different media as observed with *Ochrobactrum.* The revision and the comparison results with ATCC has been done and the

strain was identified as *Cellulosimicrobium cellulans* strain number ATCC 274

**Anatomy of Nodules**

Transverse section of nodules are shown in Fig (2), Plate 1 (a and b) for *Ochrobactrum* and plate 2 (c and d) for *Cellulosimicrobium*. The bacterial cells occupied the most central area of nodule. Also, it seems that the method of nodule invasion and formation differed from the method of rhizobia invasion.

**Degradation of Crude Oil**

The degradation capacity of the two genera indicated that the percentage of residual oil in media are 28.2% and 21.9% for *Ochrobactrum* and *Cellulosimicrobium*, respectively.


**Fig. (1). Nodules inducted by strain *Ochrobactrum* (A) and *Cellulosimicrobium* (B) in *Medicago sativa*.**

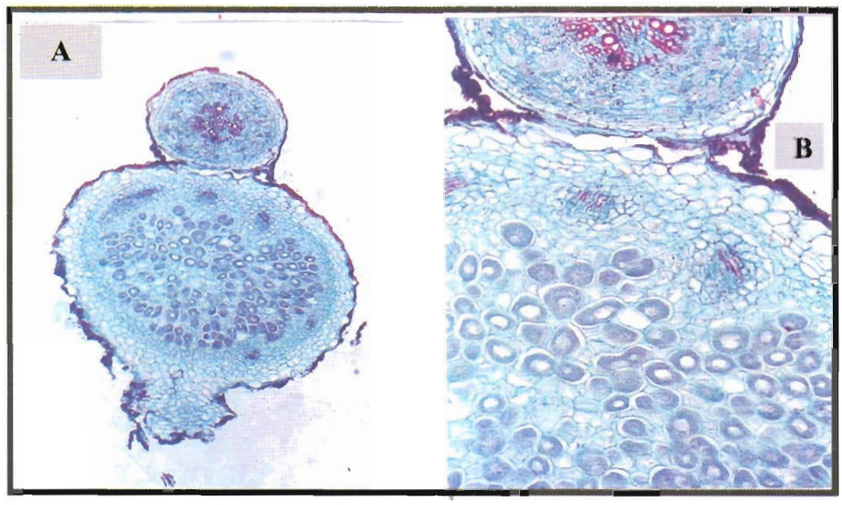


Plate (1). Transection of oval nodule formed by *Ochrobactrum*.

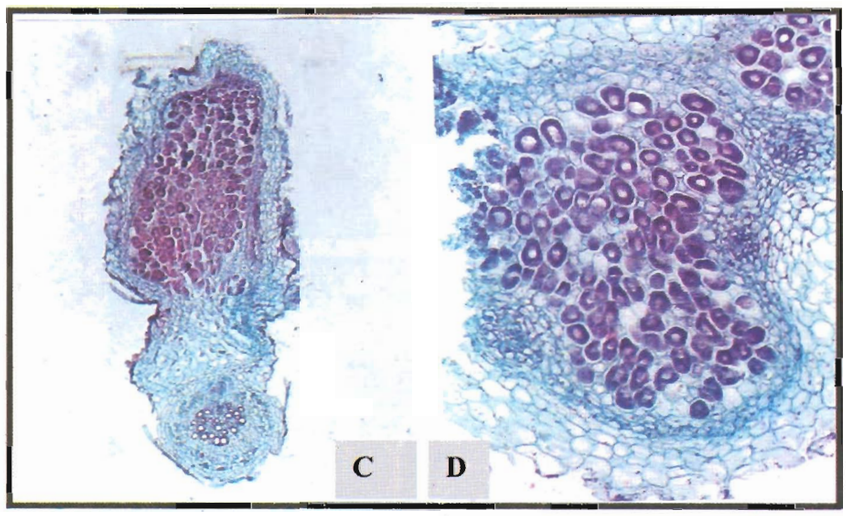


Plate (2). Transection of elongated nodule formed by *Cellulosimicrobium*

**Fig. (2). Transverse section of modules**

## DISCUSSION

Nodule formation by *Ochrobactrum* was recorded by different authors. Ngom *et al.* (2004) isolated a novel nitrogen fixing member of the *Ochrobactrum* clade from nodules of *Acacia mangium* in the Philippins and Thailand soil.

Mateos *et al.* (2005) isolated two fast growing strains, LUP21 and LUP23, from nodules of *Lupinus honoratus*. According to the rRNA gene sequences, they were identified as members of the genus *Ochrobactrum*.

There is no article discussing the capability of *Cellulosimicrobium cellulans* to form nodules on plant roots. *Cellulosimicrobium cellulans* has been regarded as a major source of yeast – lytic enzymes particularly endo – B-1,3 –gluconases, proteases and mannanase (Ferrer, 2006).

The description of *Cellulosimicrobium* is based on the description of the species *Nocardia cellulans*, and *Ochrobactrum* was initially categorized as a member of the genus *Achromobacter*. DNA-DNA hybridization and 16S sequencing data led to the foundation of the genus *Ochrobactrum* in 1988 and its partioning within the Rhizobiaceae group( Holmes *et al.*, 1988).

Another role of the two genera was observed in remediation process of hydrocarbons pollutants. Bongkeun *et al.* (2000) isolated different microbial genera capable of degrading halobenzoate from various geographical and ecological sites. The isolates were identified and classified. They were belonging to nine genera, including *Acidovorax, Azoarcus, Ochrobactrum* and others. El Shinawi and Eman (2003) isolated different genera of bacteria and actinomycetes from clay soil polluted with 10% petroleum oil. *Nocardia* , *Arthrobacter* and *Corynebacterium* were proved to be good oil degraders. Katsivela *et al.* (2004) recorded that *Ochrobactrum* isolated from refinery waste sludge posses a broad range of metabolic activities for mixtures of several classes of substrates of petroleum hydrocarbons ,such as monoaromatic and polycyclic aromatic hydrocarbons. Yoshida *et al.* (2005) studied the bacterial communities in crude oil samples from Japanese oil stockpiles, among the predominant bacteria detected in the crude oil were *Ochrobactrum anthropi, Burkholderia cepacia* and *Propionibacterium*. Bossert and Bartha (1989) recorded that *Pseudomonas, Achromobacter* and *Nocardia* were the most active bacterial species in the degradation of hydrocarbons in soil, while *Arthrobacter* and *Corynebacterium* were proved to be good oil degraders.

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# تأثير تلوث التربة بزيت البترول على تكوين العقد الجذرية فى نباتات البرسيم الحجازى

**مرفت أحمد طاهر أمارة**
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فى تجربة أصص لدراسة استخدام البرسيم الحجازى فى معالجة الأراضى الملوثة بزيت البترول الخام بمعدلات ٤% و ٨% و ١٠% تم التوصل الى النتائج التالية:

١- نمت النباتات بصورة جيدة عند كلا من تركيز ٤% و٨% بينما تدهورت النباتات المنزرعة عند تركيز ١٠%.

٢- ظهرت اشكال تعقدية على جذور البرسيم المنزرع فى التربة الملوثة بتركيز ٨% بعد ٤٢ يوم من الزراعة وتميزت بعددها القليل والحجم الكبير مع وجود نوعين منها احدهما ذا شكل بيضاوى والآخر ذا شكل عصوى.

٣- تم عزل سلالتين من الميكروبات من كلا النوعين من العقد وتم تنقيتها وتعريفها بطريقة BIOLOG وعرفت العزلة المأخوذة من العقد البيضاوية بأنها تنتمى لجنس *Ochrobactrum* والعزلة المأخوذة من العقد العصوية تنتمى لجنس *Cellulosimicrobium*

٤- للتأكد من ان تلك العزلتين لها دور فى تكوين العقد الجذرية على جذور نبات البرسيم تم تلقيح بذور البرسيم المعقمة بالعزلتين كل على حدى وزراعتها فى أرض رملية معقمة وبعد ٤٢ يوم من الزراعة لوحظ تكون العقد بنفس الشكل.

٥- تم دراسة كفاءة العزلتين فى تكسير زيت البترول معمليا واوضحت النتائج ان الزيت المتبقى بعد التكسيرفى حالة سلالة *Ochrobactrum* يعادل ٢٨,٢١%. ومع سلالة *Cellulosimicrobium* يعادل ٢١,٩%

مما سبق يتضح أن هناك اجناس ميكروبية أخرى لها القدرة على تكوين العقد الجذرية على النباتات البقولية غير بكتريا الريزوبيا. كما يتضح أن زيت البترول يحتوى على مجموعة من الميكروبات لها ادوار فسيولوجية وبيئية هامة.