

EFFICIENCY OF TWO BACTERIAL STRAINS, *Bacillus subtilis* BS2 AND *B. mycooides* BM7, ISOLATED FROM PETROLEUM - POLLUTED LANDS FOR BIOREMEDIATION OF HYDROCARBONS-POLLUTED SOIL IN SAUDI ARABIA

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ABSTRACT

The efficiency of two bacterial strains, namely: *Bacillus subtilis* Bs2 and *B. mycooides* Bm7 that have been previously isolated from petroleum-polluted soil in Saudi Arabia, for bioremediation of hydrocarbons-polluted soil were studied. The results indicated that both bacterial strains played an influential role in elimination of the phytotoxicity of benzene and toluene as evidenced by the pronounced increments in percentages of grain germination and viability of emerged seedlings, chlorophylls and carotenoids pigments contents of leaves as well as the significant improvement in plant growth vigor (root length, plant height and biomass gain) of barley (*Hordeum vulgare* L.) "Madini" cultivar as compared to that cultivated in untreated hydrocarbon-polluted soil under the same environmental conditions. However, *B. subtilis* Bs2 proved by far to surpass *B. mycooides* Bm7 in its bioremediation potential.

Keywords: Petroleum, benzene, toluene, bioremediation, grain germination, growth vigor, barley, *Bacillus subtilis*, *B. mycooides*.

INTRODUCTION

Petroleum pollution is a serious obstacle to photosynthesis that affects the fundamental life and productivity of the sea (Duke *et al.*, 2000). Not all oil released from land sources is quickly washed away to sea: moreover, pipeline and oil-well accidents, unregulated industrial wastes, and leaking underground storage tanks can all permanently contaminate large areas of soil, making them economically useless as well as dangerous to the health of organisms living in and around them (El-Baz, 1992; Hashem, 1996). Biological treatment of petroleum contamination has long been accepted as a treatment technology by which petroleum and other hydrocarbons may be eliminated from the environment (Raymond *et al.*, 1967; Atlas, 1981, 1995; Hashem, 2004).

The localization of hydrocarbon-assimilating microorganisms in oil contaminated environments has received a considerable attention by many investigators because of the possibility of utilizing their degradation potential in bioremediation of petroleum contaminated soil (Inoue and Horikoshi, 1989; Utkin *et al.*, 1992; Ijah and Ukpe, 1992; Rowland and Atlas, 1995; Venosa *et al.*, 1996; Lehmann, 1998; Chaineau *et al.*, 1999; Reardon *et al.*, 2000; Lee and Lee, 2001; Rosenberg, 2006; Yakubu *et al.*, 2007; Kim *et al.*, 2008; Saadoun and Alawawdeh, 2008; Wolicka *et al.*, 2009; Yudono *et al.*, 2009).

In Saudi Arabia, biodegradation of petroleum hydrocarbons as well as bioremediation and rehabilitation of petroleum-polluted soils by using microorganisms has been an area of interest for many years (Abu-Zinada

and Sabek, 1980; Sallal, 1995; Hashem, 1995; 1996; 2004; Hashem and Al-Obaid, 1996).

In a previous communication two bacterial strains, namely: *Bacillus subtilis* Bs2 and *B. mycooides* Bm7, have been isolated from petroleum-polluted soil at the Eastern region in Saudi Arabia; both of which proved to be capable of degrading benzene and toluene *in vitro* (Al-Tuwaijri, 2009). The present study was conducted to investigate the feasibility of using these bacterial strains to remediate soil polluted with the aromatic hydrocarbons (benzene and toluene), and to elucidate the possible effects of the various treatment of soil on grain germination and growth vigor of barley plant (*Hordeum vulgare* L.).

MATERIALS AND METHODS

Bacterial strains

A vast number of bacterial strains have been previously isolated from petroleum-polluted soils at the Eastern region in Saudi Arabia. The isolates were screened for their capacity to utilize benzene and toluene as sole source of carbon and energy. Two isolates of Gram-positive endospore forming bacilli identified as *Bacillus subtilis* (strain No. Bs2) and *B. mycooides* (strain No. Bm7) exhibited the highest benzene and toluene degrading potentials (Al-Tuwaijri, 2009). The two prospective isolates were routinely cultured on nutrient agar slants and maintained as bacterial suspensions in 20% glycerol (v/v) at - 20°C. Both isolates were employed in the present study.

Soil treatment and culture conditions

Aliquot of composite soil was distributed in cloth bags (5 kg, each) and sterilized at 121°C by autoclaving on three separate occasions for 1 hour each time, with a 24 h interval between each one. Thereafter, the contents of the bags were put in plastic pots (30 cm diameter and 25 cm depth) that have been previously sterilized by dipping in 5% formaldehyde solution, and left for 4 days to be well aerated. The pots were then transferred to a glasshouse, and divided into 8 sets (5 pots each); one set was left untreated to serve as a control, another one set was inoculated with *B. subtilis* to serve as a positive control. Another 2 sets were amended with hydrocarbon; one set with benzene and the other one with toluene, to serve as negative controls.

Treatments were conducted as follow: two sets of pots were amended with benzene and inoculated with bacteria; one (5 pots) with *B. subtilis* and the other 5 pots with *B. mycooides*. The remaining 2 sets were amended with toluene and inoculated with bacteria, one with *B. subtilis* and the other with *B. mycooides*. Soil inoculation with the prospective bacterial strains (*B. subtilis* Bs2 and *B. mycooides* Bm7), was performed using 100 ml of a dense bacterial suspension (approximately $1.4-1.5 \times 10^8$ CFU/ml) of 48 h-old culture per pot. The pots of each set were placed on an assigned bench in a glasshouse and maintained at $28 \pm 2^\circ\text{C}$ and 65-70% relative humidity for 2 weeks before planting. Barley grains (*Hordeum vulgare* L.) Madini cultivar were sterilized in 2% sodium hypochlorite solution for 1 min, washed thoroughly in a series of sterile water, and transferred into a sterilized plastic tray (25x40 cm)

containing a sheet of Whatman No. 1 chromatographic paper, then watered with adequate amount of sterile water and maintained in the dark at $28\pm 2^{\circ}\text{C}$ for 24 h to verify the viability of grains to be used. Five grains were sown, at equal distances, in each pot. The pots were maintained at $28\pm 2^{\circ}\text{C}$ and 65-70% relative humidity; irrigation was carried out immediately after sowing, and every 3 days with equal amounts of sterile water. The data were collected after 15, 30 and 45 days of cultivation; involving germination rate, viability of raised seedlings, root length, shoot length, dry weight and pigment content of healthy/survived plants (April and Sisnas, 1990; Gunther *et al.*, 1996; Railley *et al.*, 1996).

Estimation of photosynthetic pigments contents

The chlorophyll was extracted from known weights of fresh leaves using dimethyl formamide according to the methods described by Lichtenthaler (1987). The absorbance of the extract was measured at 664.5 nm for chlorophyll a; at 647 nm for chlorophyll b and at 452.5 nm for Carotenoids using a spectrophotometer (Spectronic DU 640). The concentrations of chlorophylls and carotenoids were calculated according the formula given by Wellburn and Lichtenthaler (1984), as follows:

$$\text{Chlorophyll a} = 12.7 E_{664.5} - 2.8 E_{647} \mu\text{g/ml},$$

$$\text{Chlorophyll b} = 20.13 E_{647} - 4.62 E_{664.5} \mu\text{g/ml},$$

$$\text{Carotenoids} = 4.2 E_{452.5} - (0.0264 \text{Chla} + 0.426 \text{chl b}) \mu\text{g/ml}.$$

Then, each of the three fractions was calculated on fresh weight basis (as mg g^{-1} fresh wt.).

Statistical analysis

Data were analyzed following the statistical analysis system (SAS Institute, 1988). All multiple comparisons were first subjected to analysis of variance (ANOVA). Comparisons among means were made using the least significant difference (LSD) at $P \leq 0.05$ (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

The results depicted in Table 1 show that soil pollution with either benzene or toluene adversely affected germination of barley grains, and that benzene elicited stronger phytotoxicity than toluene; the percentage germination was 24-28%, and 32-36%, respectively. Moreover, 16% (in case of benzene-contaminated soil) and 12% (in toluene-contaminated soil) of the raised seedling died post emergence, and only 12% and 24% of plants remained alive up to the 45th day of planting. This is in agreement with the results of Hou *et al.* (1999) who reported that the presence of volatile petroleum hydrocarbons in soils at relatively low concentration (4000-8000 mg/kg dry soil) could severely hinder seed germination and result in poor plant establishment. The impact of soil pollution with petroleum hydrocarbons on seed germination and emergence of seedlings has been attributed to their phytotoxicity and/or physical impeding of water and oxygen transfer between the seed and surrounding soil environment, thus hindering the germination response (Adam and Duncan, 2002).

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The results herein reported (Table 1) revealed that bacterial treatment of soil contaminated with benzene or toluene (using *B. subtilis* Bs2 or *B. mycooides* Bm7) resulted in elimination of the deteriorative effect of these petroleum products, as evidenced by the pronounced increase in percentage germination and viability of seedlings.

Table 1 further show that *B. subtilis* seemed to have higher bioremediation potential than *B. mycooides*; the percentages germination in benzene- and toluene-contaminated soils were 76 and 80% following treatment with *B. subtilis* Bs2, whereas in case of *B. mycooides* Bm7, the corresponding percentages of germination were 52 and 60%, respectively, throughout the experimental period. It may be interest to recall here that in a previous *in vitro* study *B. subtilis* Bs2 was found to exhibit higher hydrocarbon-degradation potential than *B. mycooides* Bm7 though both strains proved to be capable of utilizing benzene and toluene as sole source of carbon and energy (Al-Tuwaijri, 2009). The influential role of microorganisms in bioremediation of petroleum-polluted soil has been authenticated by many investigators (Aronstein *et al.*, 1991; Hashem and Al-Obaid, 1996; Alexander, 1999). Hanson *et al.* (1997) have shown that the treatment of petroleum-polluted soil with *Acinetobacter* sp. A3 permitted better germination and growth of mungbean (*Vigna radiate* L.). These authors deduced that this bacterium was capable of reducing crude oil phytotoxicity through biodegradation.

The results presented in Figure 1 demonstrate that the root length of barley plants survived in soil amended with benzene or toluene decreased by about 92% of the control value throughout the various intervals. This is in conformity with the results of Petukhov *et al.* (2000) who reported that petroleum hydrocarbon pollution elicits phytotoxic effect on some plants like *Avena sativa* L., *Secale cereale* L. and *Hordeum vulgare* L., as evidenced by inhibition of seed germination, reduction in the total biomass and the length of the roots. The results herein reported (Fig. 1) further demonstrate that soil treatment with *B. subtilis* eliminated considerably the phytotoxic effects of both benzene and toluene on root length of barley; wherein the root length reached 75 and 85% of the control value, respectively, after 45 days. Soil inoculation with *B. mycooides* Bm7 elicited, however, lesser bioremediation efficiency than *B. subtilis*.

The results in Figure 2 show that shoot length of barley plants survived in benzene- or toluene-polluted soil was greatly reduced compared to control. Treatment with either bacterial strains resulted in well marked increments in plant height, thus the shoot length reached 69-75% of the control value following treatment with *B. subtilis*, whereas treatment with *B. mycooides* enhanced shoot length of plants grown in toluene-polluted soil but was ineffective in case of plants grown under the impact of benzene.

Prolongation of growth period had insignificant effect on plants grown under the impact of hydrocarbon pollution, whereas the shoot lengths of plants grown in unpolluted soil (control) or in polluted soil treated with bacteria exhibited progressive increase ($P < 0.05$) by increasing the plant age up to the 45th day.

The remarkable improvement in plant growth as indicated by the progressive increase in root length and plant height in response to bacterial treatments may be attributed to the biodegradation of petroleum hydrocarbons by the employed bacterial strains to non toxic intermediates (Atlas, 1995; Banke *et al.*, 2000).

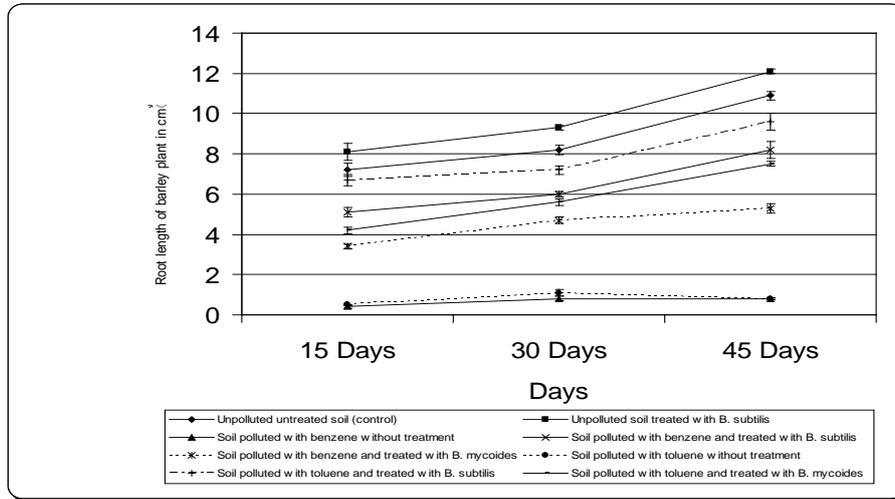


Fig. (1): Root length of barley plants grown under the impact of benzene or toluene pollution as influenced by bacterial treatment.

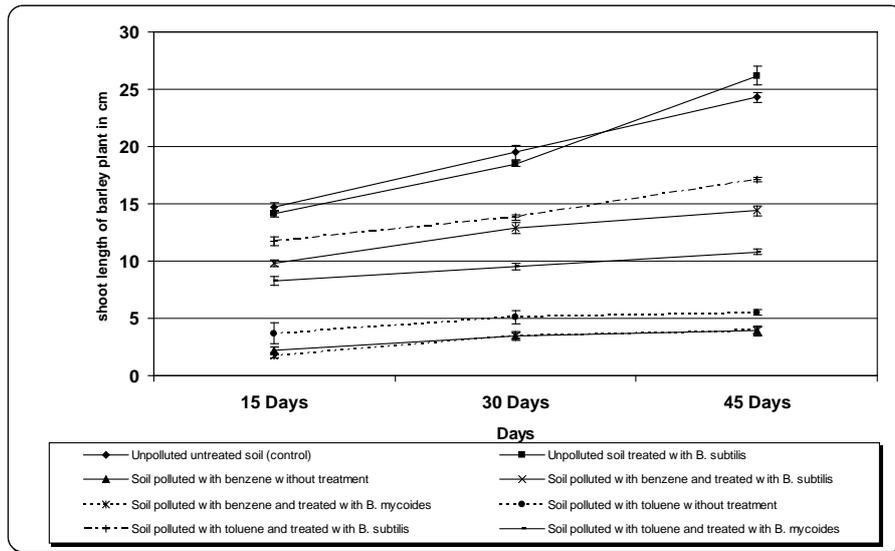


Fig. (2): Shoot length of barley plants grown under the impact of benzene or toluene pollution as influenced by bacterial treatment.

The dry weight of barley plants exhibited similar response to soil pollution by benzene or toluene, as well as to the bacterial treatments (Fig.3). Thus, the dry weight of plants survived in polluted soil was greatly reduced; being about 13% of the control value in soil polluted with benzene and 15% in toluene-polluted soil, whereas the dry weight of plants grown in polluted soil treated with *B. subtilis* reached 40% (in case of benzene) and 47% (in case of toluene). However, soil treatment with *B. mycooides* resulted in lesser biomass gain, being 21% of the control value after 15 days and reached 35% on the 45th day.

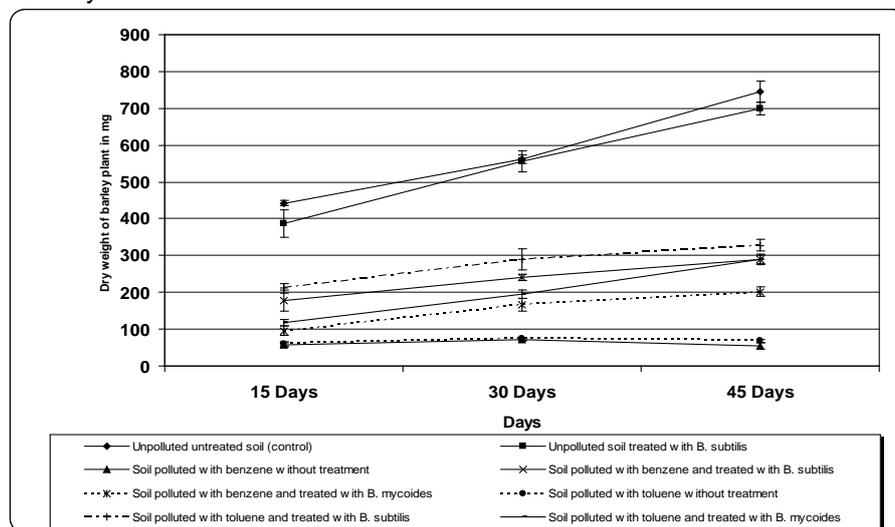


Fig. (3): Dry weight of barley plants grown under the impact of benzene or toluene pollution as influenced by bacterial treatment.

The response of photosynthetic pigments contents of barley leaves to the impact of benzene and toluene in untreated or in bacterial-treated soil is presented in Figure 4. It is apparent that both hydrocarbons caused highly significant decreases in chlorophylls and carotenoids contents of leaves as compared to control during the first 15 days. Thereafter, chlorophyll a content continued to decrease up to the 45 days, whereas chlorophyll b and carotenoid contents were not significantly affected by prolongation of growth period. It is possible that the effect of petroleum hydrocarbons on chlorophylls and carotenoids is an attenuation of the biosynthetic rate rather than a breakdown of pigments already formed. In case of plants grown in polluted soil treated with either *B. subtilis* or *B. mycooides*, chlorophyll a contents and consequently the total pigments were increased considerably by time.

It has been reported that petroleum hydrocarbon pollution may affect plants by retarding seed germination and reducing plant height, biomass and photosynthetic rate or resulting in complete mortality (Mendelssohn *et al.*, 1990; Lin and Mendelssohn, 1996; Pezeshki *et al.*, 2000). In the present study most reductions in growth and viability of barley plants due to petroleum hydrocarbons occurred within the early stages of growth particularly in root length.

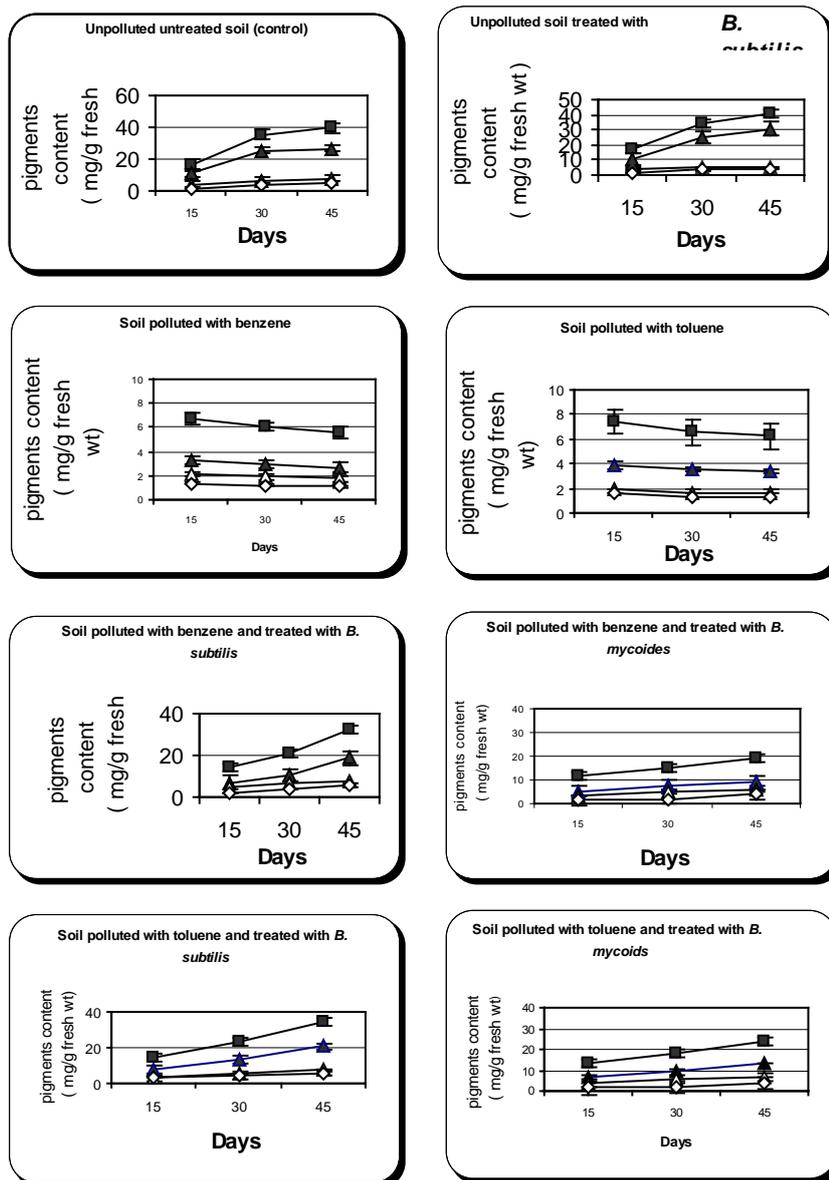


Fig. 4. Pigments contents of leaves of barley plants grown in unpolluted soil (control) and in hydrocarbon-polluted soil untreated or treated with the experimental bacterial strains.

- = total pigments ; LSD = 0.266, 0.163, 0.517, at 15, 30 & 45 days, respectively
- ▲-----▲ = chlorophyll a ; LSD = 0.253, 0.155, 0.444, at 15, 30 & 45 days, respectively.
- △-----△ = chlorophyll b ; LSD = 0.230, 0.140, 0.506 at 15, 30 & 45 days, respectively.
- ◇-----◇ = carotenoids ; LSD = 0.447, 0.275, 0.894 at 15, 30 & 45 days, respectively.

This might point to the higher sensitivity of roots than shoots, and consequently some (8-12%) of the emerged seedlings (about 40-50% of the control) died within the first 15 days. This might imply that these petroleum substances affect plant growth mainly through their adverse effect on water uptake and transport of nutrients from root to shoot.

Achuba (2006) reported that the chlorophyll contents of the leaves of 12-day-old seedlings of cowpea (*Vigna unguiculata*) were decreased by the various sublethal concentrations of crude oil. Petroleum pollution is a serious obstacle to photosynthesis. Duke *et al.* (2000) and Duke and Walkinson (2002) have demonstrated that oil contamination of sediment resulted in albinos seedlings of *Avicennia marina*; some plants die and others survive and have possible sub-lethal responses.

The present investigation clearly established the fact that *Bacillus subtilis* Bs2, and to lesser extent, *B. mycooides* Bm7 play an influential role in bioremediation of petroleum-polluted soil irrespective of the observed reduction in growth vigor of barley plants as compared to the plants grown in unpolluted soil (control). In this connection, it is worth to mention that Mergesin and Schinner (1997) stated that the hydrocarbon content of soil cannot be diminished to zero and always remain between 10-30%. This may lend a plausible explanation to our findings. Nevertheless, the results herein reported may emphasize the influential role of the experimental bacterial strains (*Bacillus subtilis* B52 and *B. mycooides* Bm7) in bioremediation of hydrocarbon- polluted soil in Saudi Arabia.

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**كفاءة سلالتين من البكتيريا - باسيلس ساتلس " بي إس 2" و باسيلس ميكويدس " بي إم 7" - المعزولة من الأراضي الملوثة بالنفط على العلاج الحيوي للتربة الملوثة بالنفط بالمملكة العربية السعودية
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تمت دراسة كفاءة سلالتين من البكتيريا، هي : باسيلس ساتلس " بي إس 2" و باسيلس ميكويدس " بي إم 7" ، المعزولة من بعض الأراضي الملوثة بالنفط بالمنطقة الشرقية بالمملكة العربية السعودية، على العلاج الحيوي للتربة الملوثة بالهيدروكربونات البترولية. وقد أظهرت النتائج أن كلتا السلالتين تلعب دورا مؤثرا في إزالة التأثير السام لكل من البنزين والبطولوين، حيث تبين أن معالجة التربة بكل منهما يؤدي إلى زيادة ملحوظة في معدل إنبات الحبوب وحيوية البادرات المنبثقة وإلى زيادة معنوية في محتوى الأوراق من الأصباغ الكلوروفيلية و الكاروتينية، كما يؤدي إلى تحسن معنوي في نمو النبات والمتمثل في الزيادة المعنوية في طول الجذر وطول الساق و الوزن الجاف لنبات الشعير (صنف مديني) ، علي أن السلالة المستخدمة من بكتيريا باسيلس ساتلس " بي إس 2" كانت أكثر كفاءة علي المعالجة الحيوية للتربة من سلالة باسيلس ميكويدس " بي إم 7".

قام بتحكيم البحث

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**أ. د/ محمود محمد عوض الله السواح
أ. د/ فتحي عواد منصور**

Table 1: Response of grain germination and viability of emerged seedlings of "Madini " barley cultivar to soil pollution with petroleum hydrocarbons and treatment with *B. subtilis* Bs 2 and *B. mycooides* Bm7

Treatment	Plant growth intervals (days)								
	15			30			45		
	Grain Germination %	Seedlings died Post emergence %	Number of survived Seedlings %	Grain Germination %	Seedlings died Post emergence %	Number of survived Seedlings %	Grain Germination %	Seedlings died Post emergence %	Number of survived Seedling %
Unpolluted untreated soil	96	-	96	96	-	96	96	-	96
Unpolluted soil + <i>B. subtilis</i>	96	-	96	96	-	96	96	-	96
Soil polluted with bebzene	24	12	12	28	16	12	28	16	12
Soil + benzene+ <i>B. subtilis</i>	76	4	72	80	8	72	80	8	72
Soil + benzene + <i>B. mycooides</i>	52	12	40	52	12	40	52	12	40
Soil polluted with toluene	32	8	24	36	12	24	36	12	24
Soil+ toluene + <i>B. subtilis</i>	80	4	76	80	4	76	80	4	76
Soil + toluene + <i>B. mycooides</i>	56	8	48	60	8	32	80	8	52

