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Effect of diesel contamination and diesel degrading bacteria on *Zea mays* growth and degradation of diesel oil

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Abstract

Soil pollution by oil compounds is one of the most common environmental problems. To study the effects of four different treatments of soil diesel pollution and diesel degrading bacteria (control, diesel, one bacterium and three bacteria) on some growth and biochemical factors of two cultivars of *Zea mays* (704 and 640) a factorial research was undertaken in the form of a completely randomized design with four replications in 2014. In addition, microbial parameters and gas chromatography analysis of plant and soil samples were performed. The results showed that in the presence of diesel contamination, dry weight (83%), leaf area (61%), chlorophyll content (71%) and relative water content (17%) of both cultivars decreased in comparing to the control plants. In all treatments, the amount of malondialdehyde (50%), flavonoids (70%), catalase enzyme (83%) and EC (35%) increased in comparing to the control. According to the results of GC analysis, the highest degradation of diesel oil in soil was related to the consortium bacteria. Researches have indicated that collaboration between plant and bacteria can speed up the removal of oil pollution from the soil.

Keywords: Bioremediation, Degrading bacteria, Diesel, Oil pollution, *Zea mays*

Introduction

Soil pollution by oil compounds is one of the most common environmental problems. Bioremediation is an important tool to reduce environmental pollution. For the first time Adam and Duncan considered the role of phytoremediation in refining oil hydrocarbons for diesel fuel effect on germination of cotton (Adam and Duncan, 2002).

Bioremediation cooperation between plant roots and soil organisms help to break down persistent organic pollutants. Some microorganisms are capable to bioremediation of diesel oil. Collaboration between plants and bacteria increase the rate of oil removal from the soil (Luepromchai *et al.*, 2007).

According to previous studies, the grasses have been mentioned as suitable plants in soil decontamination and phytoremediation because of their extensive root system. The roots cause an increase rhizosphere in soil, which increases the microbial populations in the rhizosphere and is the result of cooperation between bacteria and plants to remove oil pollution and reduce them from soil (Gudin and Syratt, 1975). *Zea mays* root systems as well as a soil amendment associate with a group of microorganisms such as Bacteria, Fungi, Actinomycetes, protozoa and mites (Wellhausen, 1952).

Some microorganisms are capable in bioremediation of gasoline. Among bacteria, Pseudomonas species have been the most extensively studied due to their superior performance in degrading a wide range of aromatic compounds (Cao *et al.*, 2009; Dindar *et al.*, 2015).

Environmental pollution from oil activities in a major oil producing country as Iran is inevitable (Hassanshahian, 2014). Therefore, survey of the effect of oil-contaminated soil on physiological and enzymatic parameters is very important for improving the yield and quality of growing green cover plants and developing the oil-rich region. Accordingly, in this research, the application of diesel degrading bacteria to promote *Zea mays* growth and reduction of diesel pollution of soil were examined by considering to the existence of extensive root system in *Zea mays*.

Materials and Methods

This research was conducted in the Laboratory of Plant Physiology, Faculty of Sciences, Yasouj University in spring of 2014. The experiment was a completely randomized factorial design with four replications. After preparation seeds of *Zea mays* cultivars (704 and 640) from agricultural service center and their disinfection by sodium hypochlorite, they were transferred to a sterile

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Petri dish containing distilled water. Germination began after three days, and after 5 days, seedlings were transferred to pots (four seedlings per pot).

Pots containing soil were divided into four groups. **The first group:** Soil pollution-free diesel was prepared without addition of bacteria, after 5 days, *Zea mays* seedlings were transferred to these pots with four replications. The second group: Seedlings were planted 1 Kg of soil with 1% (w/w) diesel. The third group: A buffer containing bacteria (*Peseudomonas fragi)* was also added to the soil in addition to 1% (w/w) diesel. The fourth group: in addition to 1% (w/w) diesel, mixture of three bacterium including, *Peseudomonas fragi***,** *Peseudomonas aeruginosa* and *Achromobacter denitrificans* were added to the soil. All treatments were maintained under a 16 hrs. dark and 8 hrs. light photoperiod at 15-20°C and were grown for 28 days in these conditions. The diesel imbued soil mechanical method was performed so that 14 ml diesel was added drop by drop to the soil texture in each pot containing one kilogram of soil separately and resulting mixture was kept for 48 hours in the dark at 25°C and then was transferred to the pots.

Total count of heterotrophic and diesel oil degrading bacteria in soil of plant treatments:

For enumeration of heterotrophic and diesel oil degrading bacteria, cells present in the soil samples were serially diluted and plated (100 µl) on Nutrient Agar (NA) and Bushnell Hass agar (BH) media, respectively. All plates were incubated at 30±1°C. After two days, the numbers of grown colonies were counted (Hassanshahian *et al.,* 2013). Results were expressed as colony forming units per one gram of soil (CFU g^{-1}).

To measure the dry weight of roots and shoots, roots, stems and leaves were harvested after washing; sections relating to a special paper bag were placed inside each pot and allowed to dry in an oven for 48 hours at 70°C. Then, envelopes were taken out from the oven and dry weights of different plant parts were measured with a balance scale model Te313s gr.

The empirical relationship of leaf area was calculated by using the following equation:

$LA = L \times W \times 0.75$

Where L is length of leaf and W is the width of the leaf.

In this method, after removing all the leaves from the stems, 1/3 of leaves of different size (small, medium and large) were randomly chosen, the largest length and width (in cm) of leaf were measured and thus leaf area was calculated.

Malondialdehyde (MDA) contents were measured using a thiobarbituric acid reaction (Heath and Packer, 1969). About 0.5 to 1.0 g of tissue was homogenized in 5 ml of 5% (w/v) tricloroacetic acid and the homogenate was centrifuged at 12000g for 15 mins. at room temperature. The supernatant was mixed with an equal volume of thiobarbituric acid (0.5% in 20% [w/v] trichloroacetic acid) and the mixture was boiled for 25 mins. at 100°C, followed by centrifugation for 5 min at 7500g to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the A600. MDA contents were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The amounts of photosynthetic pigments (chlorophyll a, b) were determined according to the method of Lichtenthaler (1987). The samples (0.25 g) were homogenized in acetone (80%) and the extract was centrifuged at 3,000g and absorbance was recorded at wavelengths of 646.8 and 663.2 nm for chlorophyll assay by a spectrophotometer (S/No80604-UV-2100). Chl a and Chl b were calculated using the following formulas (Lichtenthaler, 1987):

Chl a = (12.25 A663.2 – 2.79 A646.8)

Chl b = (21.21 A646.8 – 2.79 A663.b)

To determine the absorption by flavonoids, about 0.1 to 0.5 g of fresh leaf tissue was homogenized in 5 ml of acidic ethanol (99:1 Ethanol: Glacial Acid Acetic) and the homogenate was centrifuged and stored in warm water (80ºC) for 10 mins. Then absorbance of the supernatant was measured at 270, 300 and 330 nm (Krizek *et al.,* 1998).

The activity of catalase (CAT) was estimated by monitoring the decrease in absorbance of H_2O_2 within 30 s at 240 nm. The assay solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2 and 100 µl enzyme extract (Dhindsa and Motowe, 1981).

The electrolyte leakage (EL): Fresh leaf samples were cut into discs of uniform size (5 mm diameter) and placed in test tubes containing 10 ml of double distilled water. The tubes were incubated in a water bath at 32°C for 2 hrs. and the initial electrical conductivity of the medium (EC1) was measured. Samples were heated at 100°C for 20 mins. to release all electrolytes, cooled to 25°C and the final electrical conductivity (EC2) was measured (Sairam *et al.*, 2002). The electrolyte leakage (EL), expressed in % of total electrolytes, was calculated by using the formula:

EL = (EC1/EC2) x 100.

Leaf relative water content (RWC): Thirty leaf discs (1.0 cm diameter) were sampled and immediately weighed (FW). Next, they were immersed in distilled water in Petri dishes for 7 hrs. at 25°C under a photon flux density of 40 µmol $m^{-2}s^{-1}$, blotted on filter paper, and the turgid weight (TW) determined. Discs were dried in an oven at 80°C for 48 hrs. and the dry weight (DW) obtained. The RWC was calculated using the equation (Silveira *et al*., 2003):

$RWC = (FW-DW)/(TW-DW)\times 100$.

The soluble and insoluble sugars were determined spectrophotometrically by the phenol sulfuric acid method (Chapin and Kennedy, 1987).

After the end of experiment period (28 days), plant roots were removed from the soil. After washing, the roots of plants were separated and dried at room temperature. Residual diesel oil in soil and root samples were extracted at room temperature on a shaking table

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by using dichloromethane (CH_2Cl_2, Sigma-Aldrich, Milan; 10% v/v). This procedure was repeated three times, and the CH_2Cl_2 phase was combined and treated with sodium sulphate anhydrous (Na_2SO_4, Sigma-Aldrich, Milan) in order to remove any residual. Quantitative analysis of hydrocarbons was analysed by a GC-FID Varian 3800 model, (USA) equipped with a SE-54 capillary column (25 m X 0.32 mm) and flame ionization detector (FID). Helium was used as the carrier gas (30 ml min^{-1}).

Statistical analysis: All the data were statistically analyzed using two-way model 1 ANOVA, with treatment and cultivar as main factors. Mean values were compared using the SPSS (22 version) statistical program. Duncan tests were performed when significant differences occurred at 5% level. All results were reported as the mean ± standard error (SE).

Results and Discussion

84.08%, 81.63% and 59.18% reduction in dry weight compared to the control plants were observed for shoot dry weight of 704 cultivar by using the treatment of 1% diesel without degrading bacteria, the third treatment of exposing to diesel and bacteria (K*) P. fragi* and also the treatment of diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. Dentrificans*, respectively. 83.89%, 79.66% and 52.11% reduction in dry weight compared to the control were measured for 640 cultivar under the diesel treatment, under the treatment with diesel and bacteria (K*) P. fragi* and under the treatment of diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans*, respectively (Fig 1 a and b).

It was observed that phytotoxicity can cause such a reduction in dry weight and it was found that presence of diesel at the growth environment of trees, causes drought, yellowing and burning of the vegetative organs, especially the leaves (Palmorth and Pichtel, 2002).

The results of the current study showed that presence of oil contaminants in soil can cause production of toxic compounds and reduce plant growth that were consistent with study of Gafari Rahbar *et al.* (2012).

The amount of leaf area in the 704 and 640 cultivars in the presence of 1% diesel without degrading bacteria treatment reduced by 64.60% and 61.61%, in the presence of diesel and bacteria (K) reduced by 56.37% and 52.82% and under the fourth treatment reduced by 34.16% and 26.88%, respectively, compared to the control (Fig 2 a).

Likely the reduction of leaf area due to the decrease in relative water content and subsequently shrink cell size, reduced meristem cell division and resulting in the slow growth of leaves, it stops leaf production, accelerated aging and subsequently, the leaf fall (Lobato *et al.,* 2008; Osuagwu *et al.,* 2010). Researchers emphasized that oil pollution of soil affects physiological processes such as leaf initiation, leaf area expansion, photosynthetic ability and root activity (Agbogidi *et al.,* 2007; Peretiemo-Clarke and Achuba, 2007).

MDA content of 704 cultivar in the presence of 1% diesel and without degrading bacteria increased by 33.86%, in the presence of diesel and bacteria (K) *P.fragi* increased by 28.19% and also in the treatment diesel and in the presence of bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans* increased by 23% compared to the control. However, MDA content of the 640 cultivar under the second, third and fourth treatments increased by 50.26%, 39.05% and 15.93%, respectively, compared to the control (Fig 2 b).

Evidence suggests that MDA is a breakdown product of unsaturated fatty acids that is used as a biological marker to measure lipid peroxidation (Mittler, 2002). Increasing the level of MDA and other aldehydes in the treated plants probably are increased reactive oxygen species (ROS) and decreased antioxidant defense (Nakano *et al.,* 2002).

Chlorophyll a of 704 cultivar in the presence of 1% diesel without degrading bacteria decreased by 17.59%, in the presence of diesel and bacteria (K) *P.fragi* decreased by 7.95%, and under the treatment of diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans* showed 5.30% reduction compared to control. However, the amount of chlorophyll a in the 640 cultivar under the second, third and fourth treatments decreased by 71.12%, 51.25% and 31.12%, respectively, compared to the control (Fig 3 a).

Chlorophyll b of 704 cultivar reduced by 29.83%, 23.91% and 4.38 under treatment of 1% of diesel and without degrading bacteria, under treatment of diesel and bacteria (K) *P. fragi* and in the presence of diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans*, respectively, compared to the control. Amount of Chlorophyll b of 640 cultivar under the second, third and fourth treatments decreased by 56.26 %, 33.08% and 13.15%, respectively, compared to the control (Fig 3 b).

The cause of pigment decrease can be attributed to the induced hydrolyze and inhibit synthesis. One of the main reasons for the decrease in chlorophyll is that it was damaged by reactive oxygen species (O_2^-, H_2O_2, .OH) (Navari-Izzo *et al.,* 1990). Decreasing activity of photosystem II and Rubisco and also inhibition of ATP synthesis are causes oxygen as a substitution acceptor for additional electrons in photosynthesis and the formation of free oxygen species in chloroplasts increase (Lawlor and Cornic, 2002).

Obtained results showed that flavonoid content in shoots of both 704 and 640 cultivars in all three wavelengths increased in the presence of 1% diesel and without degrading bacteria compared to the control, it also increased under the third treatment, however it reduced in comparison with the second treatment. Moreover, it showed an increase and decrease under the treatment of diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans* compared to the control and other treatments, respectively.

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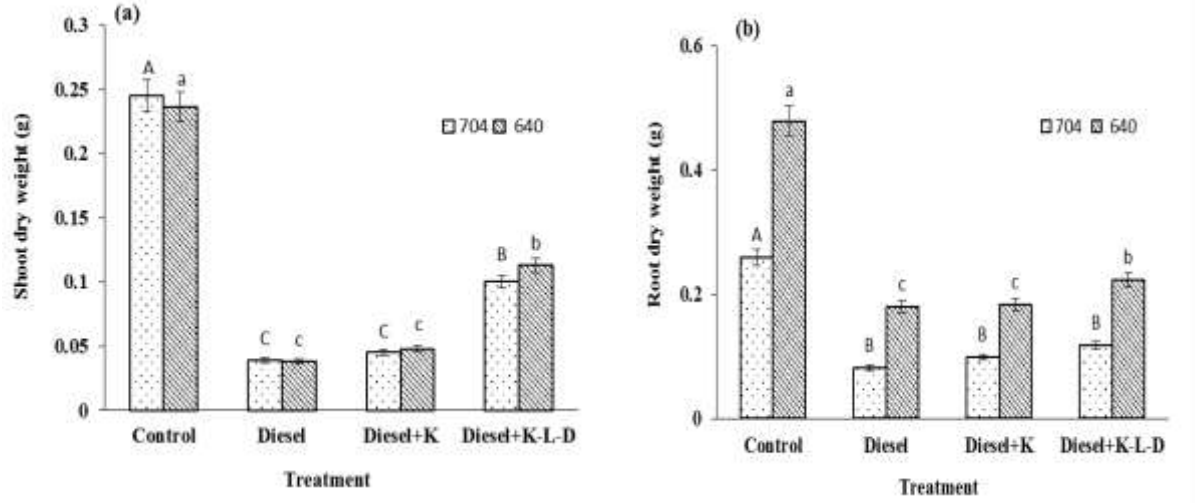

Figure 1. Effects of diesel (1% concentration) and diesel degrading bacteria on shoot (a) and root (b) dry weight of 704 and 640 cultivars.

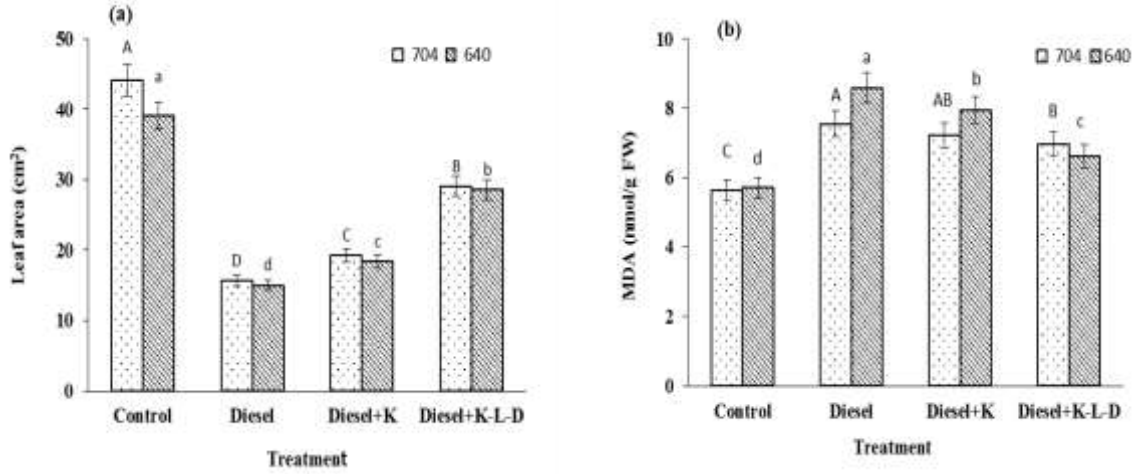

Figure 2. Effects of diesel (1% concentration) and diesel degrading bacteria on leaf area (a) and Malondialdehyde (MDA) (b) of 704 and 640 cultivars.

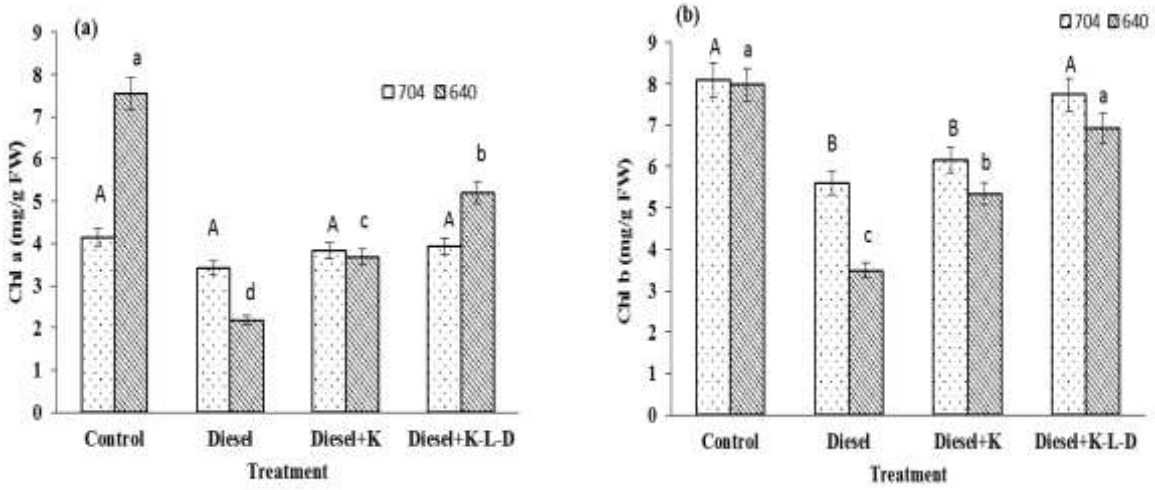

Figure 3. Effects of diesel (1% concentration) and diesel degrading bacteria on chlorophyll a (a) and b (b) in 704 and 640 cultivars.

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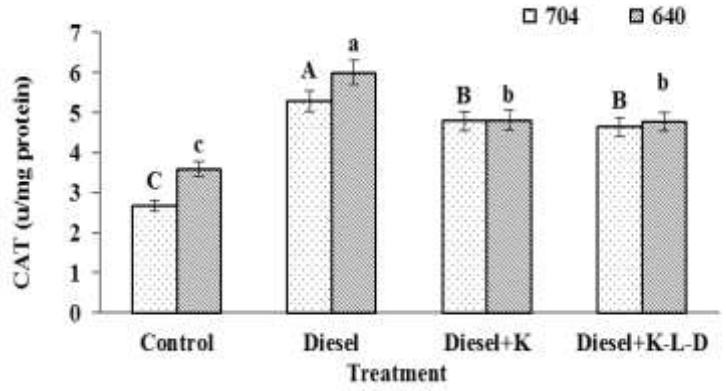

Figure 4. Effects of diesel (1% concentration) and diesel degrading bacteria on catalase activity in 704 and 640 cultivars.

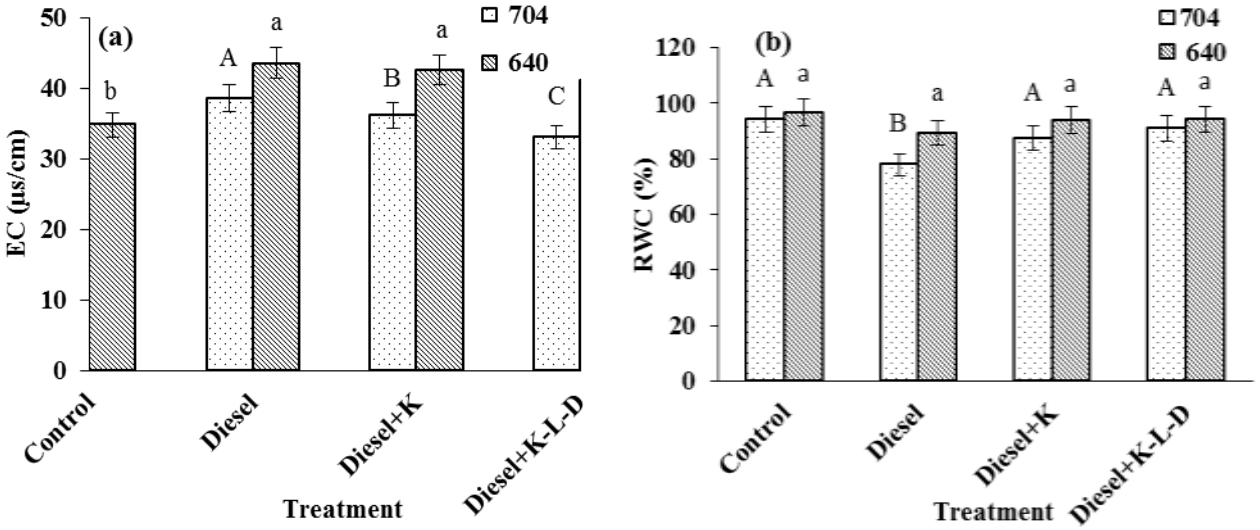

Figure 5. Effects of diesel (1% concentration) and diesel degrading bacteria on Electrolyte leakage (a) and Leaf relative water content (b) in 704 and 640 cultivars.

Flavonoids are synthesized in the cytoplasm and the cytoplasmic surface of the endoplasmic reticulum, and with antioxidant activity against biotic and abiotic stress, have protective role. Furthermore, flavonoids act as scavengers of free radicals such as reactive oxygen species (ROS), and also prevent their formation by chelating metals (Pourcel *et al.,* 2006).

Catalase activity of 704 cultivar in the presence of 1% diesel and without degrading bacteria increased by 97.7%, and in the presence of diesel and bacteria (K) *P. fragi* increased to 79.10% and under the treatment of diesel and bacteria (K, L, D*) P. fragi, P. aeruginosa, A. dentrificans* increased by 73.88% compared to the control. However, catalase activity in 640 cultivar under the second, third and fourth treatments increased by 67.3%, 34.10% and 32.82%, respectively, compared to the control (Fig 4).

Plant cells are protected against oxidative damages by equipping them with a scavenger system of free radicals includes antioxidant enzymes such as catalase and peroxidase (Cho and Park, 2000).

Electrolyte leakage of plasma membrane of 704 and 640 cultivars in the presence of 1% of diesel and without degrading bacteria increased by 35.34 and 24.94%, in the presence of diesel and bacteria (K) *P. fragi* increased by 27.06 and 22.18% and in the presence of diesel and bacteria (K, L, D*) P. fragi, P. aeruginosa, A. dentrificans* increased by 15.98 and 18.08%, respectively, compared to the control (Fig 5 a). Mallalah and Afzal (1996) believe that tension effects of organic pollutants on plants cause changes in the amyloplast membrane and electrolyte leakage.

Leaf relative water content of 704 cultivar in the presence of 1% diesel and without the presence of degrading bacteria decreased by 17.15%, in the presence of diesel and bacteria (K) *P. fragi* decreased to 7.03% and under the treatment with diesel and bacteria (K, L, D) *P. fragi, P. aeruginosa, A. dentrificans* decreased by 3.22 % compared to the control. However, leaf relative water content of 640 cultivar under the second, third and fourth treatments decreased by 7.68, 2.86 and 2.62%, respectively, compared to the control (Fig 5 b).

Soil moisture treatment with hydrocarbon loss due to hydrophobic properties which, lead to the loss of water and food stuffs (Chaineau *et al.,* 1997), that in addition

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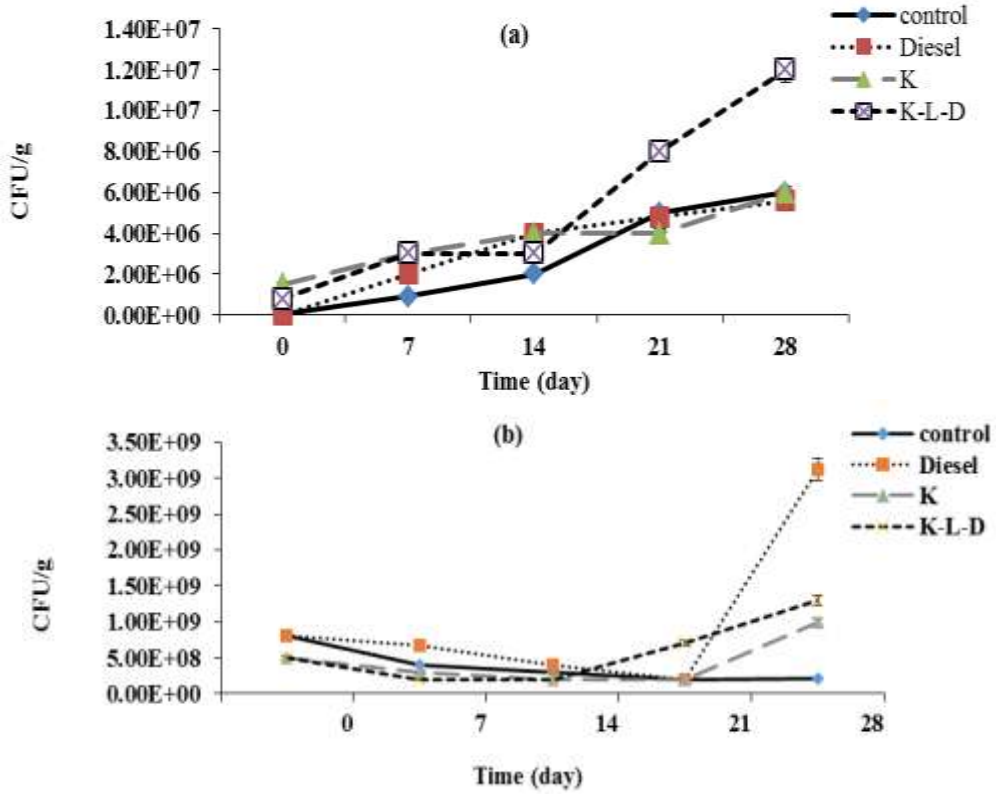

Figure 6. Changes in the number of degrading (a) and heterotrophic bacteria (b) (CFU) in the rhizosphere of *Zea mays* in the treatment of concentration (1%) and diesel degrading bacteria in 5 steps (0, 7, 14, 21, 28 day)

to the stress of organic pollutants can cause drought stress to the plants (Kaya *et al.*, 2006).

Leaf carbohydrate content of 704 cultivar under the second, third and fourth treatments increased by 96.20%, 67.08 and 39.24%, respectively, compared to the control. However, leaf carbohydrate content in 640 cultivar under the second, third and fourth treatments increased by 92.85, 38.57 and 31.42%, respectively, compared to the control.

Increased sugars in plants under stress is a defense mechanism that through osmotic regulation, prevents the destruction of enzymes and clear hydroxyl radicals, increases the tolerance of plants against stress (Kuznetsov *et al.*, 1997). According to Bates *et al.* (Bates *et al.*, 1973) reasons for this increase in the stress, are likely associated with free radicals production and the various forms of active oxygen. This could be caused by oxidative stress, which endangers the life of the cells. Therefore, plants response to this type of stress by increasing the levels of soluble sugars.

The quantity of diesel oil degrading bacteria was determined in all treatments. As shown in Fig 6 a, the number of diesel oil degrading bacteria have increment pattern in all treatments. The highest quantity was observed on day 28 Th. in the fourth treatment (1.20E + 07 CFU g^{-1}).

The quantity of heterotrophic bacteria was determined in all treatments. The results were shown in Fig 6 b. As shown in this figure the number of heterotrophic bacteria decreased dramatically until 21 days after treatment. But in the twenty-first day showed a rising trend. From twenty-one to twenty-eight days of experiment increment pattern were observed for heterotrophic bacteria as the maximum amount of these bacteria were found at the end of the experiment (28 day) (3.12E + 09 CFU g^{-1}).

According to the results, the reduction of heterotrophic bacteria quantity suggests that diesel oil have toxic and inhibitory effects on the population of soil heterotrophic bacteria. But over time, the number of heterotrophic bacteria increased, because the heterotrophic bacteria gradually adapt to the diesel pollution. Studies on population trend of degrading and heterotrophic bacteria showed that after addition of petroleum, hydrocarbons can increase the number of degrading bacteria (Margesin *et al.*, 2000). Diesel oil degrading bacteria use these organic pollutants as source of carbon and energy. The biodegradation of these pollutants by bacteria take place in aerobic condition and in the presence of oxygen. For example in the aerobic degradation of polycyclic aromatic hydrocarbons (PAHS) bacteria use dioxygenase enzyme. This enzyme incorporated two oxygen atoms into hydrocarbon molecules and decreased toxicity of compounds such as acids, CO_2 and water (Eweis *et al.*, 1998; Pothuluri and Cerniglia, 1994).

Degradation of the diesel oil in three treatments was examined by Gas Chromatography (GC-FID); the concentration of diesel oil was calculated in soil and root separately. The results, expressed as percentage of diesel oil degradation, showed that soil with consortium of K, L and D bacterial strains had 87% degradation

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although only 63% of diesel oil degradation observed for soil with single bacterium (K strain). In the roots of plant with consortium bacteria 76% of degradation was recorded and only 48% of degradation were calculated for roots with single bacterium. These results confirmed that the highest degradation of diesel oil at soil were observed for consortium bacteria (Soil+K,L,D).

This study has demonstrated that diesel oil has a significant effect on the growth of *Zea mays*. The results related to this research confirmed that the collaboration between diesel oil degrading bacteria and rhizosphere of *Zea mayes* can dramatically decrease diesel oil contamination and we can use this technology for remediation of diesel polluted soils.

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