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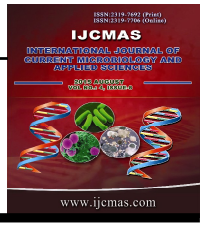

Original Research Article

The Effect of Steam Sterilization of a Petroleum-Contaminated Soil on PAH Concentration and Maize (*Zea mays* L.) Growth

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Keywords

Polycyclic aromatic hydrocarbon, Autoclaving, Petroleum contaminated soil, Rhizotron, Soil chemical properties

A B S T R A C T

Sterilized soil is often used as a control to distinguish between biotic and abiotic reactions and in petroleum-contaminated soils it is used to verify bioremediation experiments. In the present study we performed a laboratory and a greenhouse experiment to evaluate the effect of steam sterilization, e.g. autoclaving, on some of the chemical properties of a petroleum-contaminated soil as well the effects on shoot and root growth of maize (*Zea mays* L.). The results showed that the autoclaving significantly decreased pH and increased the amounts of electrical conductivity and available phosphorus in both contaminated and uncontaminated soils. However total nitrogen, organic carbon and cation exchangeable capacity statistically remained constant. Sterilization also decreased total Zn, Ni and Mn in uncontaminated soil and total Fe in petroleum contaminated soil. Almost all polycyclic aromatic hydrocarbons (PAHs) were strongly increased in contaminated sterilized soil in compared to the non-sterilized one. The total concentration of PAHs increased around 250% as affected by sterilization treatment, while the content of total petroleum hydrocarbon (TPH) in the sterile soil was almost similar to the non-sterile soil. Growing parameters of maize such as root and shoot biomass, shoot height, total root length, number of root tips and root depth were strongly decreased in the contaminated soil. Furthermore sterilization of uncontaminated soil had a positive effect on shoot biomass while it was slightly negative for the contaminated soil. Shoot dry weight of maize planted in uncontaminated sterile soil was about 30% higher than in uncontaminated non-sterile soil. Therefore it is important to consider both the changes of soil properties and petroleum compounds and effects of these alterations on the plant growth when studying autoclaving sterilized soils.

Introduction

Soil sterilization is often used in plant-growth experiments to clearly distinguish the biotic and abiotic processes which occur in soil (Wang *et al.*, 2011) and it is to

eradicates, inhibits or controls any major class of soil borne organisms such as weeds, nematodes, fungi and bacteria (Razavi Darbar and Lakzian, 2007). The most common of soil sterilization methods include γ-irradiation, amendment with poisons (e.g. $HgCl_2$, NaN_3), ultraviolet, chloroform fumigation, microwave irradiation and autoclaving (Paguirigan and Beebe, 2007; Razavi Darbar and Lakzian, 2007). Autoclaving is a steam sterilization method for eliminating the viable organisms in soils. Due to its low cost and ease of use, autoclaving is a commonly used method for sterilizing soil. Autoclaving kills soil organisms by raising temperatures to 121 °C and at 103.4 kPa. The duration of autoclaving depends on the amount of soil, therefore higher amount of soil need longer time to be used (Wang *et al.*, 2011). Trevors (1996) suggested that the minimum autoclaving time for inhibition of microbial activity in soil was 20 to 30 min at 121°C and 100 kPa. It has been assumed that 20 to 30 min is enough for sterilizing soil in the study of polycyclic aromatic hydrocarbon (PAHs) degradation (Yu *et al.*, 2005; Wang *et al.*, 2008).

The expectation is that the sterilization while eliminating all viable organisms should cause no significant effects on soil properties. But it has been reported that all known sterilization methods commonly cause some secondary effect on soil and alter its physical and chemical properties (Jenneman *et al.*, 1986; Anderson and Magdoff, 2005; Razavi Darbar and Lakzian, 2007; Berns *et al.*, 2008; Sinegani and Hosseinpur, 2010; Wang *et al.*, 2011).

Although, autoclave sterilization effects on the properties of unpolluted soil have been the aim of several studies (Razavi Darbar and Lakzian, 2007; Bank *et al.*, 2008; Berns *et al.*, 2008; Sinegani and Hosseinpur, 2010), but according to our knowledge there is no report regarding the effect of autoclave sterilization on the properties of petroleum-contaminated soils as well on the plant growth.

Thus, a petroleum contaminated and an uncontaminated soil were used to investigate the effect of autoclave sterilization method on the chemical properties of these soils as well the concentration of polycyclic aromatic hydrocarbons in the contaminated soil. In addition, we performed a rhizotron experiment to evaluate the effect of the autoclaved sterile soil on the root and shoot growth of maize (*Zea mays* L.). The hypothesis was that sterilization of petroleum contaminated soil with steam, alters soil properties and increases the amounts of PAHs compounds during the autoclaving and consequently would affect the plant growth.

Material and methods

Soils sampling and sterilization

Contaminated and uncontaminated soils (loamy sand, Typic Torriorthents) were collected from a landfill nearby the Shahid Hasheminejad Gas Refinery Complex at Sarakhs in Northeastern Iran. The site has an arid climate with a long-term mean temperature of 18.8 °C and an annual rainfall averaging about 180 mm. The soil was contaminated due to the diesel-contaminated effluent which produced during the gas extracting. Three samples were collected from topsoil (0 – 30 cm depth), bulked, thoroughly mixed, air-dried and sieved through a mesh of 2 mm size. Uncontaminated soil was also collected in the vicinity of the landfill. The concentration of total petroleum hydrocarbons (TPH) in the contaminated soil averaged 21.6±0.6 mg g^{-1}.

About 7 kg of each contaminated and uncontaminated air-dried soils were individually sterilized in an autoclave for 20 minutes at 121 °C and 100 kPa. This procedure was repeated twice with a 24-hour incubation period at room temperature in between. Immediately after autoclaving, soil samples were taken and stored at 4 °C until the analysis time. Soil chemical properties were determined by standard methods.

Rhizotrons, soil packings and plant cultivation

The rhizotrons had a wooden frame and back plate and a removable front cover made of a 4 mm thick glass plate. Figure 1 shows a schematic view and dimensions of the rhizotron used in this study. The inner space of the rhizotrons was 30 cm high, 20.5 cm wide and 1.5 cm thick. The front glass plate was covered with an opaque black plastic to prevent light entering except for the times of observation.

The four soil packing treatments of the rhizotrons (Fig. 2) were: (I) petroleum-contaminated non-sterile soil, (II) petroleum-contaminated sterile soil, (III) uncontaminated non-sterile soil and (IV) uncontaminated sterile soil. The packing were constructed layer by layer using the same filling procedure in all four cases, only the origin of soil was varied with respect to the treatment as described above. The contaminated soil was covered with a 2.5 cm layer of uncontaminated soil (sterile or non-sterile) in order to facilitate plant establishment (Kechavarzi *et al.*, 2007). The four treatments were replicated three times.

Maize (*Zea mays* L. cv. SC704) seeds were continuously surface-sterilized by rinsing in 70% ethanol (30 sec), in 5% sodium hypochlorite (NaOCl) (5 min) and five times briefly with sterilized distilled water. After vernalization at 4 °C, the seeds were placed on agar surface medium in closed Petri-dishes and incubated for 2 days at 25 °C to germinate. The germinated seedlings with radicles of about 1 cm length were selected for the experiment. Single seedling was then planted at a depth of 1cm in the uncontaminated top soil layer in the center of each rhizotron. All rhizotrons were placed in a glasshouse (temperature 28±4 °C, day/night cycle 13/11 h, and a 48±7% relative humidity) in a randomized array. The rhizotrons were placed on a rack with a 45° inclination to induce roots growing along the front glass to enable visual growth monitoring. Soil moisture was kept approximately constant (near 70% field capacity) by periodical watering in order to replace consumed water.

According to the Kechavarzi *et al.* (2007) experiment, root development was recorded periodically 12, 16, 22, 26, 33 and 45 days after transplanting by tracing all roots visible through the front glass on acetate transparencies. The transparencies were then scanned at 300 dpi to obtain digitized images and analyzed for parameters such as total root length, number of root tips and depth of rooting using the Smart Root plugin of the software package ImageJ (Lobet *et al.*, 2011). At the same dates, also the shoot height of each seedling was measured using the soil surface as a datum. Immediately after the last recording, 45 days after transplanting, the experiment was terminated. Roots and shoots were separated after harvesting, weighed and oven-dried.

Extraction and quantification of TPH and PAHs

Soxhlet was used to extract petroleum hydrocarbons from homogenized soil samples (10 g) in a 1:1 (v/v) mixture of HPLC-grade dichloromethane and n-hexane

(125 ml). The extracts were sequentially purified and cleaned up using silica gel 60 (0.063–0.200 mm, Merck) to adsorb the polar compounds. The residues obtained (and weighed) after evaporation of the solvents in a rotary evaporator were considered as total petroleum hydrocarbons (TPH) according to USEPA (1998). The 16 USEPA target PAH were determined in the extracts by means of high-performance liquid chromatography (HPLC) using a KnauerC18-S5ODS1 column (250 mm×4.6 mm) and spectrophotometric UV detection at λ= 280 nm. The mobile phase contained methanol with a 1.5 mL/min flow rate.

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) in combination with the t-test and post-hoc analysis of Least Significant Differences. All statistical analyses were performed by means of the statistical software package SAS-Version 9.1.3 (SAS Institute, 2005).

Results and Discussion

Chemical characteristics of soils

Generally, chemical properties were affected by sterilization procedures in both petroleum-contaminated and uncontaminated soils (Table 1). The autoclaving significantly decreased the amounts of pH in both contaminated and uncontaminated soils (0.27 and 0.12 units, respectively). Electrical conductivity was in average about 30% higher in autoclaved soils than in controls and this increment was about 11% for total nitrogen (insignificant). The amount of available phosphorus (P) in petroleum-contaminated and uncontaminated soils was significantly increased by autoclaving. The concentration of available P in contaminated sterile soil were 138% higher than in the contaminated non-sterile soil, while this comparison in uncontaminated soil was just 21%. The results also showed that the sterilization treatment have no significant effect on the amounts of organic carbon and cation exchangeable capacity of both uncontaminated and contaminated soils.

The total amounts of trace elements as affected by autoclaving are also shown in table 1. The changes of trace elements were mostly observed in uncontaminated soil. The results showed that the autoclaving significantly has decreased the amounts of Zn, Ni and Mn in uncontaminated soil, however with the exception of Fe, all measured trace elements in contaminated soil as affected by sterilization treatment were statistically constant. The amount of total Fe in contaminated sterile soil was significantly about 30% lower than in contaminated non-sterile soil.

The concentration of hydrocarbons in petroleum-contaminated soil

The results (Table 2) show that autoclaving strongly affected the concentrations of polycyclic aromatic hydrocarbons in the contaminated soil. While the content of total petroleum hydrocarbon (TPH) in the sterile soil was almost similar to the non-sterile soil, the total concentration of PAHs (selected 16 PAHs) increased around 250% as affected by sterilization treatment. With the exception of benzo(a)anthracene, the concentration of all measured PAHs in the sterile soil were higher than in the non-sterile soil, however the increment in the concentration of chrysene and benzo(g,h,i) perylene were not significant. The concentration of benzo(a)anthracene in sterile soil was significantly decreased around 50% in compared to the non-sterile soil.

The highest alterations of the 16 target PAHs as affected by sterilization treatment was happened for fluoranthene and naphthalene in which the concentration of these compounds in sterile soil was respectively 1286 and 1113% higher than in the non-sterile treatment. Also the concentration of the prominent carcinogenic fraction (e.g. benzo(a)pyrene) was increased more than 100% in sterile soil as compared to the non-sterile soil. Also, the concentration of benzo(b) fluoranthene, benzo(k) fluoranthene, dibenzo(ah) anthracene and indeno (1,2,3-cd)pyrene in the sterile treatment were 2.3, 2.4, 5.1 and 7.2 times higher than in the non-sterile one.

Plant growth in the rhizotrons

The effects of the different treatments on root and shoot dry weight of the maize plants are shown in figure 3. Soil petroleum contamination significantly ($P < 0.05$) decreased shoot and root biomass. The lowest plant biomass was observed in the rhizotrons of treatment II, which filled with contaminated sterile soil and the highest biomass was in the rhizotrons of treatment IV, which filled with uncontaminated sterile soil. The results showed that autoclaving had a negative effect on the biomass of maize planted in petroleum contaminated soil and it was positive for maize planted in uncontaminated soil, however the statistical analysis of the data showed that the differences of root and shoot dry matter yield in the sterile and non-sterile treatments were just significant between shoot dry weights of uncontaminated treatments, e.g. treatment III and IV. Shoot dry weight of maize planted in treatment IV was about 30% higher than in treatment III.

Figure 4 shows that the treatments with petroleum contamination strongly inhibited the development of maize roots. Total root length, root depth and number of root tips in the contaminated treatments were significantly lower than in the uncontaminated ones. For instance, total root length, root depth and number of root tips of maize planted in contaminated treatments at the end of experiment were in average 87, 83 and 82% lower than in the uncontaminated treatments. Sterilization treatment did not have significant effect on root development parameters of maize, nevertheless autoclave sterilization slightly decreased these parameters in contaminated treatment as well slightly increased them in uncontaminated soil. Figure 4 provides further evidence of the strong inhibitory effect of the petroleum contaminants on shoot growth of maize. Shoot height growth of maize at the end of experiment in the treatment IV, as the lowest ones, was 55% less than in the treatment II, as the highest ones. In contrast to the root growth parameters which have not been significantly affected by autoclaving, shoot height trends towards to be affected by sterilization treatment for most of the times particularly at the final days of the experiment.

Steam sterilization (e.g. autoclaving) by decreasing population of soil microorganisms, and changes some of the chemical properties, affects the growth of higher plants in soil. Studies have reported different results about the effect of soil sterilization by autoclave on the soil chemical properties. For example, Berns *et al.* (2008) observed that soil pH after autoclaving was remained unchanged in a Gleyic Cambisol however it was decreased in Orthic Luvisol, but Bank *et al.* (2008) found no changes in soil pH due to autoclaving. Decreasing in soils pH may have resulted from solubilization of organic acids and the magnitude of the change depended on buffering capacity of the soils.

Razavi Darbar and Lakzian (2007) who studied the effect of different soil sterilization methods on chemical properties reported that all the sterilization methods caused a significant increase in electrical conductivity and autoclaving had the highest influence on this parameter. They expressed that the increasing of soil electrical conductivity after autoclaving may be due to release ions in the soil solution with cleaving the bonds of humic substances and residual killed biomass. Our results showed that there was no significant difference between autoclaved and non-autoclaved in terms of the amounts of soil organic carbon and total nitrogen, however other fractions of these compounds must be studied. It seems, the changes in soil organic matters as affected by autoclaving, depends on soil type. Sandler *et al.* (1988) reported that autoclaving caused no significant effect on the organic matter of Evesboro (loamy sand) and Matapeake-Newark (silt loam) soils, however this process increased the amount of organic matters of Pocomoke soil (loamy sand) and decreased this parameter in Matapeake-Dover (silt loam) and Sassafras (loamy sand) soils. In many studied soils, high temperature heating or autoclaving showed an increase to the amount of extracted available P (Serrasolses *et al.*, 2008). High temperature and pressure during soil autoclaving convert phosphorus in complex organic compounds and microbial cells into more available components like as orthophosphate (Anderson and Magdoff, 2005) as well release of inorganic phosphorus by killed microbial cells (Serrasolsas and Khanna, 1995). Therefore, more increasing in available P of petroleum contaminated soil as affected by autoclaving could be the result of more complex organic compounds and cleavage of this compound in this soil.

Our results showed a decrease on some of total amount of trace elements but due to lack of sufficient information, we had no precise assumption for the changes of total amount of trace elements with autoclaving in soils, but it seems that heating and pressure treatment during the autoclaving, changed the total amount of elements probably due to destruction or alteration of the elements in the soil. Mortality of soil microorganisms, solubilisation of organic matter in soil and chemical changes in soil components could be the main reason of changes in soil properties after steam sterilization (Serrasolses *et al.,* 2008).

The composition of petroleum is highly complex and high temperature and pressure in the steam sterilization process can also alter its composition in soil. Cleavage of the aromatic rings and carbon chains in soil may occur with autoclaving. More low-molecular-weight compounds in the autoclaved soil cause more phytotoxicity effects. Additionally, sterilization by autoclave increased the concentration of the carcinogenic compounds in the petroleum contaminated soil that they are too toxic to humans and other organisms associated with the soil. There have been no researches evaluating the effects of soil sterilization methods, such as autoclaving, on the availability and concentration of the PAHs in contaminated soil, therefore further studies in this area can be helpful.

The results confirmed the previous studies that soil petroleum contamination can inhibit growth of plants (Merkl *et al.,* 2005; Besalatpour *et al.,* 2010; Masakorala *et al.,* 2013; Hentati *et al.,* 2013; Noori *et al.,* 2014). Phytotoxic effects of petroleum hydrocarbons in the soil could be the major reason of this inhibition. Inhibition of plant growth on petroleum-contaminated soil may also result from the lower water and nutrient availability due to hydrophobicity effects

(Besalatpour *et al.*, 2010). Petroleum contaminants in the soil limits the availability of nutrients to the plant for growing and physically hindering water and oxygen transfer between the seed and surrounding soil environment, thus inhibiting the growth of seedlings (Adam and Duncan, 2002). Besalatpour *et al.* (2008) reported that petroleum hydrocarbons in the soil decreased seed germination of tall fescue more than 50%. Also, Eze *et al.* (2014) who investigated the effect of different levels of light crude oil on the germination and shoot growth of maize and sorghum, reported that at the moderate levels of crude oil (e.g. 2–2.5%), seed germination of both crops was decreased however, at the high concentration of crude oil (e.g. more than 5%) seed germination of both crops was completely stopped. They also observed a decreasing in shoot height with increasing level of crude oil in the soil.

In the autoclaved soil, the plant growth may increase by improving the amount of nutrients in soil; however, the toxicity of some components would decrease plant growth in autoclaved soils. Increasing of 250% in the total concentration of PAHs in the autoclaved petroleum-contaminated soil slightly decreased the growth of maize, however this reduction would be higher if the petroleum-contaminated soil were not covered by a 2.5 cm layer of uncontaminated soil. Uncontaminated layer had allowed the plants to establish and initially grow with lower toxicity, however root development of maize was strongly limited in both sterile and non-sterile contaminated soil.

Table.1 Characteristics (mean ± standard error, n=3) of the two soils (with and without petroleum contamination) used in this study. Values with the same letters in a row for each soil are not significantly different at $P < 0.05$

Parameter	Uncontaminated soil		Contaminated soil	
	Non-sterile	Sterile	Non-sterile	Sterile
pH	7.24 ± 0.12^{A}	6.97 ± 0.05^{B}	7.42 ± 0.06^{a}	7.30 ± 0.03^{b}
EC (dS m^{-1}**)**	1.28 ± 0.05^{B}	1.66 ± 0.03^{A}	1.25 ± 0.02^{b}	1.68 ± 0.11^{a}
Organic carbon (%**)**	0.67 ± 0.03^{A}	0.67 ± 0.06^{A}	2.95 ± 0.19^{a}	2.96 ± 0.03^{a}
Total N (mg kg^{-1}**)**	135.0 ± 4.4^{A}	151.7 ± 3.9^{A}	200.0 ± 10.3^{a}	221.7 ± 7.5^{a}
Available P (mg kg^{-1}**)**	28.10 ± 0.60^{B}	34.00 ± 1.00^{A}	11.01 ± 1.22^{b}	26.20 ± 1.97^{a}
Total Fe (mg kg^{-1}**)**	181.3 ± 4.7^{A}	171.7 ± 3.2^{A}	132.3 ± 2.8^{a}	91.67 ± 4.17^{b}
Total Zn (mg kg^{-1}**)**	82.47 ± 3.99^{A}	31.33 ± 0.84^{B}	26.33 ± 0.51^{a}	28.30 ± 0.77^{a}
Total Cu (mg kg^{-1}**)**	7.47 ± 0.24^{A}	8.73 ± 0.41^{A}	3.57 ± 0.33^{a}	3.57 ± 0.06^{a}
Total Ni (mg kg^{-1}**)**	32.53 ± 1.86^{A}	22.48 ± 0.88^{B}	6.25 ± 0.40^{a}	8.83 ± 0.47^{a}
Total Mn (mg kg^{-1}**)**	199.5 ± 3.27^{A}	161.3 ± 4.7^{B}	88.67 ± 5.34^{a}	91.67 ± 4.07^{a}
CEC ($Cmol^{+}kg^{-1}$**)**	32.87 ± 1.89^{A}	32.50 ± 1.60^{A}	9.23 ± 1.02^{a}	12.43 ± 0.89^{a}

Table.2 Total petroleum hydrocarbon content and concentration of PAHs in the contaminated sterile and non-sterile soils (mean ± standard error, n=3). Values with the same letters in a row are not significantly different at P < 0.05

Compound (abbreviation, total rings number)	**Concentration (µg kg^{-1})**	
	Non-sterile	Sterile
Naphthalene (Nap, 2)	63.3±3.3^{b}	768±27.7^{a}
Acenaphthene (Ace, 3)	59.7±3.2^{b}	565±30.6^{b}
Fluorene (Flu, 3)	20.3±1.5^{b}	77.7±4.0^{a}
Phenanthrene (Phen, 3)	380.3±9.3^{b}	1423.3±29.1^{a}
Anthracene (Anth, 3)	2.1±0.3^{b}	16.0±0.9^{a}
Fluoranthene (Flt, 4)	29.3±2.0^{b}	406.7±15.0^{a}
Pyrene (Pyr, 4)	190.3±6.6^{b}	500.0±25.2^{a}
Benzo(a)anthracene (BaA, 4)	77.3±2.3^{a}	36.0±3.6^{b}
Chrysene (Chry, 4)	349.7±13.7^{a}	380.0±16.7^{a}
Benzo(b)fluoranthene (BbF, 5)	50.7±4.0^{b}	118.3±10.8^{a}
Benzo(k)fluoranthene (BkF, 5)	6.3±0.7^{b}	15.3±0.8^{a}
Benzo(α.)pyrene (BaP, 5)	19.7±1.3^{b}	41.7±1.2^{a}
Dibenzo(a,h)anthracene (dBahAn, 5)	9.7±0.7^{b}	49.7±1.2^{a}
Benzo(g,h,i)perylene (BghiP, 6)	17.7±1.3^{a}	25.7±1.5^{a}
Indeno(1,2,3-c,d)pyrene (InPy, 6)	3.8±0.4^{b}	27.3±1.3^{a}
∑PAHs	1280.2±31.2^{b}	4450.7±76.3^{a}
TPH (mg g^{-1})	21.6±0.2^{a}	21.8±0.6^{a}

Fig.1 Schematic view and dimensions of rhizotron

Dimension, mm			
	Length	**Width**	**Thickness**
Outer	310	235	23
Inner	300	205	15

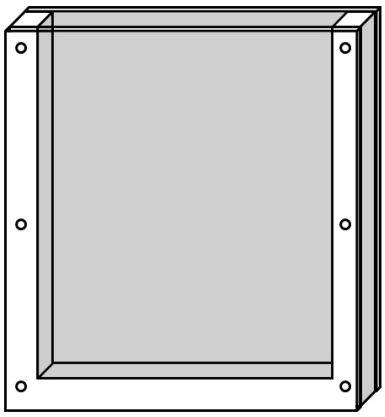


Fig.2 Schematic of the four rhizotron treatments

Fig.3 Root and shoot biomass of maize plants grown for 45 days in petroleum contaminated and uncontaminated soil. Treatment I: petroleum-contaminated non-sterile soil, Treatment II: petroleum-contaminated sterile soil, Treatment III: uncontaminated non-sterile soil and Treatment IV: uncontaminated sterile soil

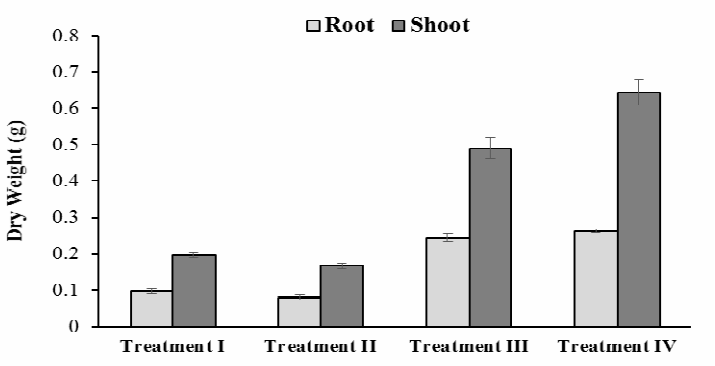
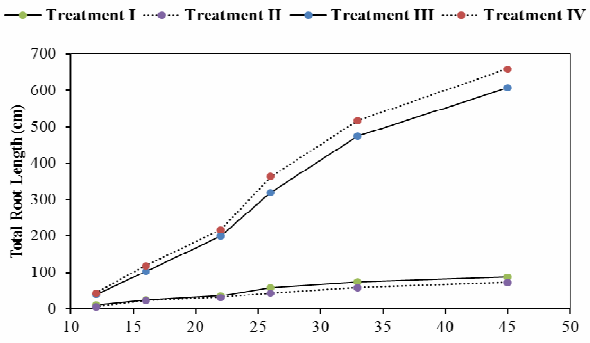
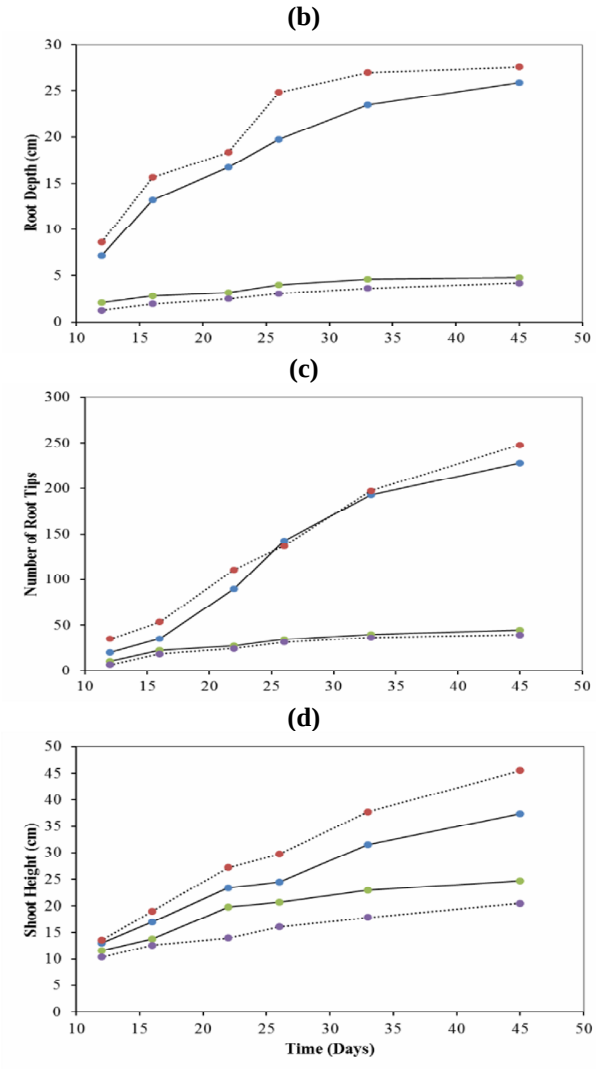


Fig.4 Development of total root length (a), root depth (b), number of root tips (c) and shoot height (d) of maize plants grown in treatment after different times of growth (12, 16, 22, 26, 33 and 45 days). Treatment I: petroleum-contaminated non-sterile soil, Treatment II: petroleum-contaminated sterile soil, Treatment III: uncontaminated non-sterile soil and Treatment IV: uncontaminated sterile soil

(a)





Autoclaving of the petroleum-contaminated and uncontaminated soils changed chemical properties of soils. Autoclaving decreased the soil pH and increased electrical conductivity and available phosphorus of both contaminated and uncontaminated soils, while sterilization caused no effect on organic carbon, total nitrogen and cation exchangeable capacity of these soils. The TPHs content of petroleum-contaminated soil in sterile and non-sterile treatments was almost constant, but the concentration of PAHs was strongly increased by autoclaving. Heating soil in the autoclave affected petroleum compounds in the soil and increased the amount of PAHs. Although soil sterilization by autoclave did not significantly affect most of the plant growth parameters but all growth parameters of maize has been decreased in the sterile petroleum-contaminated soil and increased in sterile uncontaminated soil in compared to the non-sterile treatments. This study highlighted only some consequences of autoclaving in petroleum-contaminated soil and it seems the further researches are needed to evaluate the chemical, physical and biological properties of autoclaved petroleum-contaminated soils as well to evaluate the fate of PAHs in these soils over time.

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