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EFFECTS OF COTTON ON SEVERAL ENZYMATIC ACTIVITIES OF THE PETROLEUM CONTAMINATED SOIL. A LABORATORY EXPERIMENT

A laboratory experiment lasting for 90 days was conducted to examine the interaction between a remediation plant, *Gossypium hirsutum*, and the petroleum contaminated soil. Root indices and 9 various types of soil enzymatic activities in two separate groups were determined. Results showed that compared to the uncontaminated control, the growth of cotton roots was slightly strengthened at the pollution level of 1000 mg·kg^{-1}, while seriously inhibited at the pollution level of 2000 mg·kg^{-1} and 4000 mg·kg^{-1}. At the same pollution level, all studied soil enzymatic activities except alkaline phosphatase were markedly higher in the group with plants than in the group without plants which may indicate that the content of nutrients essential for plant growth as well as the activities of microorganisms capable of degrading contaminants were both enhanced in soils planted with cotton, therefore conducive to the increase of the overall fertility level and the degradation of pollutants in the petroleum contaminated soil.

1. INTRODUCTION

The treatment of soil contaminated with petroleum in North China has always been a major and tough problem, demanding prompt solution. A variety of remediation methods, including physical, chemical and biological ones, have been employed to solve this problem. Phytoremediation, an important component of bioremediation, is a method employing living higher organisms to eliminate pollutants from environmental media. The known mechanism of phytoremediation mainly includes phytoextraction, phytovolatilization, phytodegradation and rhizodegradation [1]. Compared to traditional physicochemical methods, phytoremediation has many advantages such as lows cost, easy operation, non-destructiveness and causing no secondary pollution

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etc., in terms of in-situ treatment of contaminated soils [2, 3] and is therefore thought to be an effective and highly promising remediation technology [4]. Hence, good results may be gained through the application of phytoremediation to the treatment of the petroleum contaminated soil in North China, which has fascinated many scientists and scholars in this field.

The success of phytoremediation in removing pollutants from environmental media depends primarily on selection of plant species. Until now, numerous preliminary investigations concerning the screening of plant species for phytoremediation have been undertaken to seek out the specific ones suitable for a certain region. Our work is intended to check the efficacy and practicability of cotton (*Gossypium hirsutum*) as a remediation species. Cotton is an economic crop widely cultivated in North China and due to its biological characteristics, cotton seems to be a possible ideal species with high remediation potential. Large biomass, wide adaptability, strong viability and stress tolerance allow its application in the treatment of large-area petroleum contaminated soil in North China without any ecological risk. Thick and sturdy roots deep pushed into the soil enable it to remove pollutants accumulated in relatively deep layers. The fast growth and short duration of maturation may lead to a raise of the remediation efficiency and vigorous transpiration during the growth period is quite conducive to the gathering of pollutants, using water as a carrier, around root zones for further degradation [5–7]. Most importantly, as a fibre crop of high economic value, cotton is mainly used to make paper, cloth, rope or to serve as chemical raw materials and its products typically do not enter into the human food chain, consequently posing little threat to physical health of mankind. Therefore, it seems highly practicable to apply cotton in remediating the petroleum contaminated soil in North China for the purpose of obtaining good results both ecologically and economically. However, comprehensive studies concerning the mechanism of the interactions between cotton and petroleum contaminated soil as well as the impact brought about by this interaction on each of the two sides have rarely been performed until recently. Much further information on this subject is therefore required urgently.

Soil enzymes, important components of soil, play an important role in the mineral circulation of carbon, nitrogen, phosphorus, etc., as well as the transformation of organic matter in soil. Some important edaphic metabolic processes which include the decomposition of organic input and the detoxification of xenobiotics, are usually catalyzed by soil enzymes. Soil enzymes, which can reflect the strength and direction of various biochemical processes, are also in the central position in the soil environment and are typically taken as a sensitive indicator of the physicochemical properties of soil [8, 9]. Moreover, soil enzymatic activities, which can display the overall level of soil biological activity as well as the transforming course of soil nutrients are important indices to measure the fertility level of soil [10]. Soil enzymes are also closely related to the amount of petroleum degrading microorganisms, participating directly or

indirectly in the degradation of petroleum contaminants. Therefore, analysis, from the viewpoint of soil enzymatic activities, of the changes of soil characteristics in aspects of physicochemical properties, nutrient content, microorganic activity, etc. during the phytoremediation process contributes greatly to better elucidate the mechanism of phytoremediation for the petroleum contaminated soil. The information available at present seems quite incomprehensive, concerning mostly with effects of exogenous chemical pollution (e.g. petroleum, pesticides, heavy metals, polycyclic aromatic hydrocarbons, etc.), single or combined on soil enzymatic activities [11–14]. However studies of soil enzymes in the context of the plant–soil–petroleum contaminant complex environment and the correlation between different soil enzymatic activities under such conditions are still insufficient. More detailed information is therefore needed about the changes of soil enzymatic activities during the phytoremediation process of the petroleum contaminated soil as well as the implication manifested by these changes.

Based on the above considerations, petroleum and cotton as the target contaminant and the remediation plant have been selected to perform the laboratory experiment. The principal objective of the research is to shed some light on the interaction between cotton and petroleum contaminated soil in order to assess its significance in the treatment of petroleum pollutants. A systematic study of the growth status of cotton in the petroleum contaminated soil as well as the effects of cotton on the activities of several symbolic hydrolases and oxido-reductases in this polluted soil was conducted for a better understanding of the mechanism of the phytoremediation process of petroleum pollutants. The ecological impacts of cotton as a remediation plant on the petroleum contaminated soil in North China was also evaluated tentatively to provide fundamental data for further studies in this area.

2. MATERIALS AND METHODS

Soil treatment. The experimental petroleum was taken from Shengli Oil Field and the experimental soil was sampled from the uncontaminated 0–20 cm superficial soil layer (not detected of petroleum contaminants) in Tianjin University of Technology (39.10 N, 117.14 E). Basic physicochemical properties of the experimental soil were measured by routine methods at first [15, 16] (Table 1). Then the experimental soil was sieved (<3 mm) after being air-dried naturally in a plastic film and thereafter spiked with petroleum. The level of petroleum pollution concentration was set as follows: 0 $mg \cdot kg^{-1}$ (uncontaminated control), 1000 $mg \cdot kg^{-1}$ (low concentration), 2000 $mg \cdot kg^{-1}$ (medium concentration) and 4000 $mg \cdot kg^{-1}$ (high concentration). Spiked soil was sieved (<3 mm) again to guarantee the homogeneity of each treatment.

Table 1

Basic physicochemical properties of the experimental soil

Bulk density (dry soil) [g·cm^{-3}]	Total organic matter [g·kg^{-1}]	Total N [g·kg^{-1}]	Total P [g·kg^{-1}]	Total K [g·kg^{-1}]	pH	Mechanical composition [%]		
						Sand	Silt	Clay
1.47	11.64	0.89	0.75	3.72	7.36	26.35	41.08	32.57

Experimental design. 5 kg of the treated soil was placed in each cylindrical pot of the diameter of 20 cm, 25 cm high and then the soil was arranged to maintain the soil height in each pot at 20 cm. Treatment of each concentration level was repeated in triplicate (planted with cotton, known as the "group with plants") and at the same time each repeat was paired with the corresponding unplanted soil treatment (without any plant, known as the "group without plants") which was administered identically with the former. After that, all pots were firstly watered to full extent with distilled water and then laid aside for 3 days sheltered from wind and rain in order to make fully blended of the petroleum, soil and water to reach stable state. The moisture content of each pot soil was adjusted to 25%. Then, for groups with plants, each pot was sown with 10 pregerminated seeds of *Gossypium hirsutum* on twenty-fifth April, 2011. All pots were placed in a laboratory to create the requisite conditions for the normal growth of plants. Seedlings were thinned out after full emergence to maintain their amount at 5 plants within each pot. The temperature of the laboratory corresponded to the outdoor natural temperature and the moisture content of each pot soil was kept at 65% during the entire growth period. Necessary physical preventive measures against diseases and pests were introduced to ensure normal growth of the experimental plants. After 90 days of growth and development, all plants ceased to be watered and then the sampling and analyzing were conducted.

Sampling and analyzing. In order to collect soil samples, a small soil auger was inserted into the soil along the centre of the pot (not hurting plant roots as much as possible). Soil samples from the depth of 0–5 cm, 5–10 cm, 10–15 cm and 15–20 cm were collected for ca. 20 g of each soil layer. Each sample was placed in an individual sealed plastic bag and then stored immediately in a 4 °C refrigerator before determination its enzymatic activities. For the purpose of collecting plant samples, each plant was removed entirely from the pot (make sure to maintain the intactness of its roots and stem) and root indices such as length of the main root (average of 5 plants), total root amount (5 plants), root amount of single plant, root weight of single plant were measured and recorded.

Enzymatic activities of each soil sample were assayed according to methods previously described in literature with certain modifications. Details of assaying methods are as follows:

Activity of soil polyphenol oxidase was measured by the photometric method [17]. Pyrogallic acid was used as the substrate and the absorbance of the extract was determined at the wavelength of 430 nm. Activity of soil polyphenol phosphatase was expressed by the amount (mg) of purpurogallin produced by 1 g of dry soil after 2 h.

Volumetric method [17, 18] was used to determine the soil catalase activity which was expressed by the amount of cm^3 of 0.1 M $KMnO_4$ consumed by 1 g of dry soil after 24 h.

To determine the dehydrogenase activity of soil, a soil sample was incubated at 30 °C for 24 h. With triphenyltetrazolium chloride (TTC) serving as a hydrogen receptor, the absorbance of triphenylformazan (TPF) produced was measured at the wavelength of 485 nm and dehydrogenase activity was expressed by the amount (in mg) of TPF produced by 1 g of dry soil after 24 h [19, 20].

The lipase activity of soil was determined by the titrimetric analysis. Butyric acid released by tributyrin was extracted with ethyl acetate and quantified titrimetrically with 5 $mmol \cdot dm^{-3}$ NaOH solution. Lipase activity was expressed by the relative lipase units [21, 22].

The invertase activity of soil was determined by the colorimetric method [23, 24]. The saffron solution of 3-amino-5-nitrosalicylic acid produced underwent colorimetric determination at 508 nm and the invertase activity was expressed by the amount (in mg) of the glucose liberated by 1 g of dry soil after 24 h.

To measure the urease activity of soil, buffered urea solution added to the soil sample was incubated for 24 h at 37 °C and then its absorbance was determined colorimetrically with a spectral photometer at 578 nm. The urease activity was expressed by the amount of ammonium-N (in μg*)* released by 1 g of dry soil after 24 h [25, 26].

The protease and alkaline phosphatase activities of soil were both determined by the colorimetric method [27, 28]. The protease activity was expressed by the amount (in mg) of tyrosine liberated by 1 kg of dry soil after 1 h, the alkaline phosphatase activity was expressed by the amount (in mg) of phenol liberated by 1 g of dry soil after 24 h.

The determination of fluorescein diacetate hydrolysis (FDA hydrolysis) was carried out by the methods described by Schnürer and Rosswall [29]. 1 g of dry soil sample was placed in a triangular flask of the volume of 25 cm^3 and then 10 cm^3 phosphate buffer (pH = 7.6) and 0.1 cm^3 of FDA (2 $mg \cdot cm^{-3}$, acetone) were added. After incubation for 2 h at 25 °C, to the mixture within the triangular flask 5 cm^3 of acetone were added to terminate FDA hydrolysis. Absorbance of the solution obtained by filtrating the above mixture was determined photometrically at 490 nm. The activity of FDA hydrolase was expressed by the amount o fluorescein (in μg) released by 1 g of dry soil after 2 h.

Data processing. Each data was presented as the mean of three measurements. The method of Kolmogorov–Smirnov was adopted to check the normal distribution of the variables. Data conforming to normal distribution were implemented with the analysis

of variance (ANOVA) or paired *t*-test according to specific demand. Data not obeying to normal distribution was performed with the Kruskal–Wallis post hoc test. Standard deviation (S.D.) and the Pearson correlation coefficient were both calculated. The confidence interval of the whole data analyzed was 95%. Significance levels were $P < 0.05$ or $P < 0.01$. All statistical analyses were carried out with the IBM SPSS 13.0 software package.

3. RESULTS

3.1. EFFECTS OF PETROLEUM POLLUTION ON THE GROWTH STATUS OF COTTON ROOTS

The growth of cotton roots displayed generally a fluctuating trend at various petroleum pollution levels (Table 2). Roots grew most vigorously at low pollution concentration (1000 mg·kg^{-1}), with indices such as length of the main root, total root amount, root amount of a single plant and root weight of a single plant higher than those at medium concentration (2000 mg·kg^{-1}), high concentration (4000 mg·kg^{-1}) and uncontaminated control (0 mg·kg^{-1}).

Table 2

Root indices of cotton for various petroleum pollution concentrations

Root indices	Petroleum concentration [mg·kg^{-1})			
	0	1000	2000	4000
Length of the main root, cm	7.64 ± 4.34	9.72 ± 3.31	6.86 ± 2.35	4.93 ± 1.26
Total root number (5 plants)	54.00 ± 10.81	76.00 ± 18.37	43.00 ± 8.03	18.00 ± 5.84
Root number for a single plant	10.80 ± 4.74	15.20 ± 4.59	8.60 ± 3.17	3.60 ± 1.02
Root weight of single plant (dry weight), g	1.62 ± 0.15	2.49 ± 0.18	1.45 ± 0.12	0.97 ± 0.08

Results are presented as means ± S.D.

3.2. SOIL ENZYMATIC ACTIVITIES AT VARIOUS PETROLEUM POLLUTION CONCENTRATIONS

Oxido-reductase involved in intracellular metabolism. Polyphenol oxidase activity in the group with plants (Table 3, Fig. 1a) showed a progressively ascending trend with the increment of petroleum pollution concentration, increasing from 1.68 mg·g^{-1}·2h^{-1} (0 mg·kg^{-1}) to 2.78 mg·g^{-1}·(2 h)$^{-1}$ (4000 mg·kg^{-1}). Enzymatic activity in soil of each pollution level was higher than that in the uncontaminated control, with the difference between in medium concentration (2000 mg·kg^{-1}) and in the uncontaminated control as well as between in high concentration (4000 mg·kg^{-1}) and in the uncontaminated control reaching extremely significant level ($P < 0.01$). Compared to group with

Table 3

Soil enzymatic activities at various petroleum pollution concentrations

Enzyme type	Petroleum concentration [mg·kg^{-1}]											
	0			1000			2000			4000		
	1	2	3	1	2	3	1	2	3	1	2	3
Polyphenol oxidase	1.68±0.31	1.04±0.12	61.54^{c}	1.94±0.15	1.12±0.10	73.21^{c}	2.36±0.25	1.23±0.16	91.87^{c}	2.78±0.22	1.50±0.14	85.33^{b}
Catalase	3.69±0.21	3.34±0.17	10.48^{b}	3.72±0.19	3.29±0.32	13.07^{b}	3.85±0.16	3.28±0.12	17.38^{b}	3.47±0.20	3.34±0.17	3.89
Dehydrogenase	0.55±0.07	0.51±0.08	7.84	0.71±0.10	0.52±0.04	36.54^{c}	0.74±0.05	0.53±0.04	39.62^{c}	0.60±0.07	0.54±0.05	11.11
Lipase	4.29±0.62	2.59±0.13	65.64^{c}	5.14±0.38	3.17±0.19	62.15^{b}	6.37±0.75	3.39±0.26	87.91^{c}	7.22±0.84	3.62±0.16	99.45^{c}
Invertase	26.38±1.20	25.09±0.77	5.14^{b}	28.88±1.13	26.89±0.91	7.40^{b}	25.48±0.68	24.45±0.72	4.21	21.51±0.94	21.03±0.77	2.43
Protease	8.28±0.85	6.43±0.59	28.77^{c}	8.40±0.74	6.65±0.58	26.32^{c}	8.25±0.45	6.59±0.72	25.19^{c}	8.17±0.90	6.52±0.84	25.31^{c}
Urease	273.10±9.66	196.35±7.51	39.09^{c}	274.02±15.18	203.80±11.95	34.46^{c}	265.67±17.43	193.83±8.27	37.06^{c}	267.59±11.35	182.00±10.48	47.03^{c}
Alkaline phosphatase	3.36±0.27	4.92±0.36	–31.71^{c}	3.71±0.22	5.03±0.44	–26.24^{c}	3.81±0.42	5.22±0.31	–27.01^{c}	3.87±0.40	5.42±0.37	–28.60^{c}
FDA hydrolase	132.52±6.64	66.02±2.40	100.73^{c}	187.97±12.53	70.40±9.84	167.00^{c}	155.94±14.48	70.81±5.06	120.22^{c}	137.32±10.92	69.11±4.53	98.70^{c}

[a]Results presented in the table are obtained by paired *t*-test between enzymatic activities in group with plants (1) and enzymatic activities in group without plants (2). The difference between enzymatic activity of each soil in group with plants (1) and that in group without plants (2) with respect to (2) (in %) is given in column (3). Values of enzymatic activities are expressed by means of ± standard deviation (S.D.), their units are given in Chapter 2 (Sampling and analyzing).

[b]The significance level at $P < 0.05$.

[c]The significance level at $P < 0.01$.

plants, polyphenol oxidase activity in the group without plants exhibited a similar trend with the changes of petroleum pollution concentration (Fig. 1b, Table 3). The increased enzymatic activity in the group with plants over that in the group without plants was observed at each pollution level (Fig. 2, Table 3), with the largest margin appearing at the pollution level of 2000 mg·kg^{-1}, reaching 91.87% ($P < 0.01$).

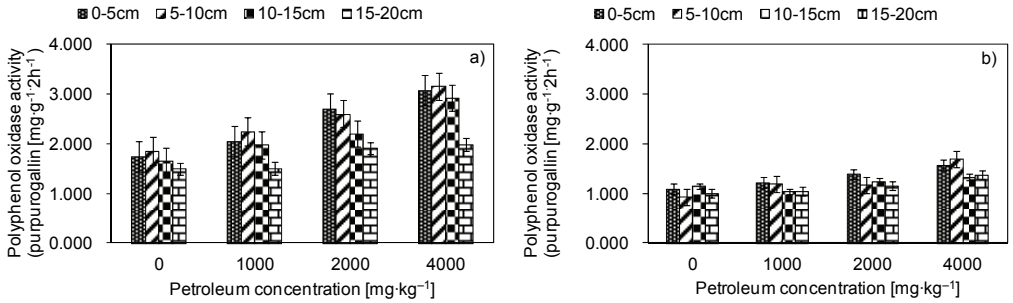

Fig. 1. Polyphenol oxidase activity for various different petroleum concentrations: a) group with plants, b) group without plants

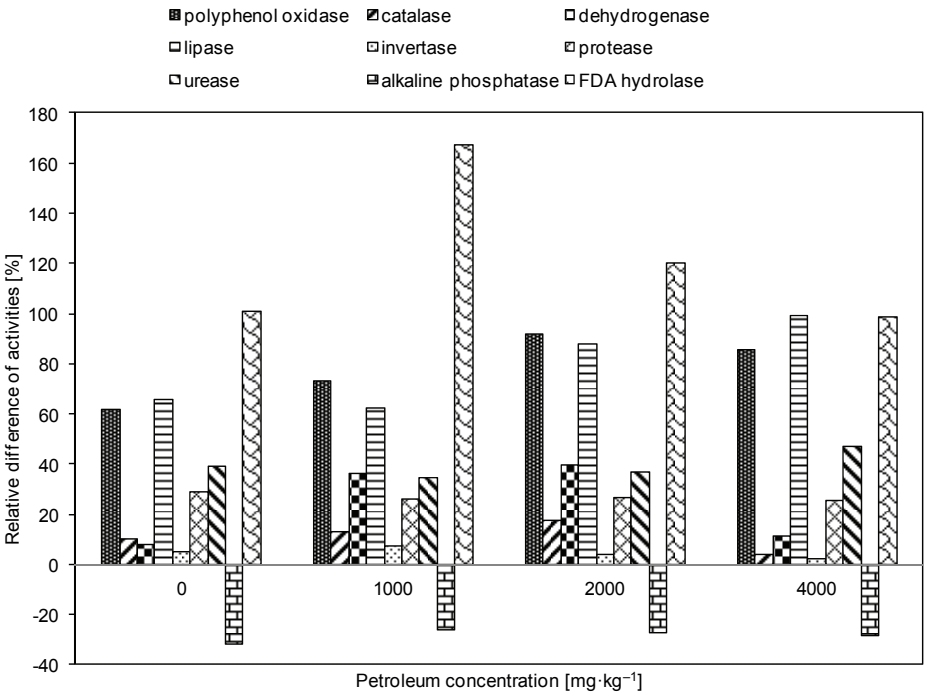

Fig. 2. Relative increase of each soil enzymatic activity in the group with plants over that in the group without plants for various petroleum concentrations

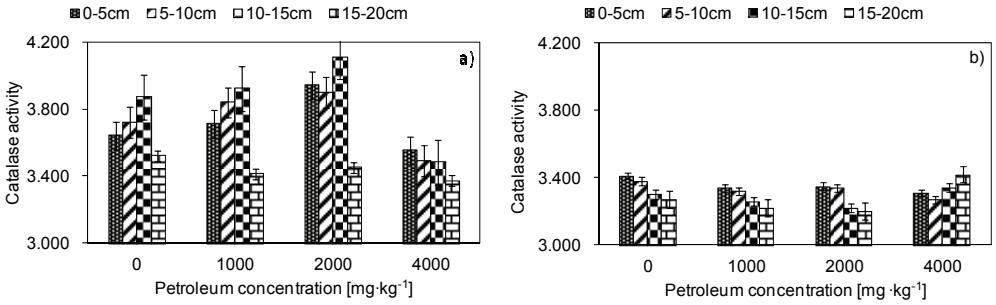

Fig. 3. Catalase activity (0.1 mol·dm^{-3} $KMnO_4$ [cm^3.g^{-1}· (24h)$^{-1}$])
for various petroleum concentrations: a) group with plants, b) group without plants

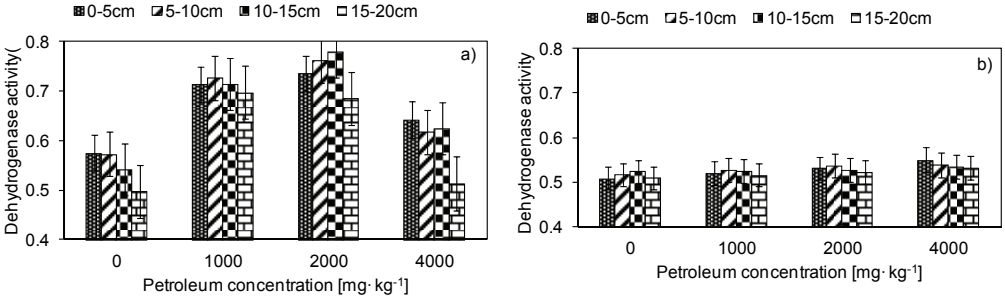

Fig. 4. Dehydrogenase activity (TPF [mg·g^{-1}· (24 h)$^{-1}$]) for various petroleum concentrations:
a) group with plants, b) group without plants

General trends of activities of catalase (Fig. 3a, Table 3) and dehydrogenase (Fig. 4a, Table 3) upon changing concentration of petroleum pollution in group with plants were similar: increase of the increment of pollution concentration first and sharp decrease afterwards. Positive correlation between activities of these two enzymes and pollution concentration were both shown up below pollution level of 2000 mg·kg^{-1}, while considerable difference between activities of these two enzymes was observed at high pollution level (4000 mg·kg^{-1}): catalase activity was already significantly lower than the uncontaminated control ($P < 0.05$), whereas dehydrogenase activity remained significantly higher than the uncontaminated control ($P < 0.05$) despite decreasing to a large extent compared to that of 2000 mg·kg^{-1}. Compared to the group without plants, activities of these two enzymes increased dramatically in the group with plants, with the largest margin both occurred at the pollution level of 2000 mg·kg^{-1}, reaching 17.38% ($P < 0.05$) and 39.62% ($P < 0.01$) (Fig. 1, Table 3). Differences of catalase activity and dehydrogenase activity among each pollution level in the group without plants were both insignificant ($P > 0.05$) (Fig. 3b, Fig. 4b, Table 3).

Hydrolase involved in the carbon cycle. Similarly as in polyphenol oxidase, there was an extremely significant positive correlation between lipase activity (Fig. 5a, Table 3) and petroleum pollution concentration in the group with plants ($r = 0.877$, $P < 0.01$), from which an extremely significant ($P < 0.01$) difference between lipase activity at the pollution level of 2000 mg·kg^{-1} and 4000 mg·kg^{-1} and that in the uncontaminated control could be revealed respectively. Similar findings could be found in the group without plants ($r = 0.879$, $P < 0.01$). At the same pollution level, lipase activity was considerably higher in the group with plants than that in the group without plants, the increase margin both exceeding 80% ($P < 0.01$) at the pollution level of 2000 mg·kg^{-1} and 4000 mg·kg^{-1} (Fig. 1, Table 3).

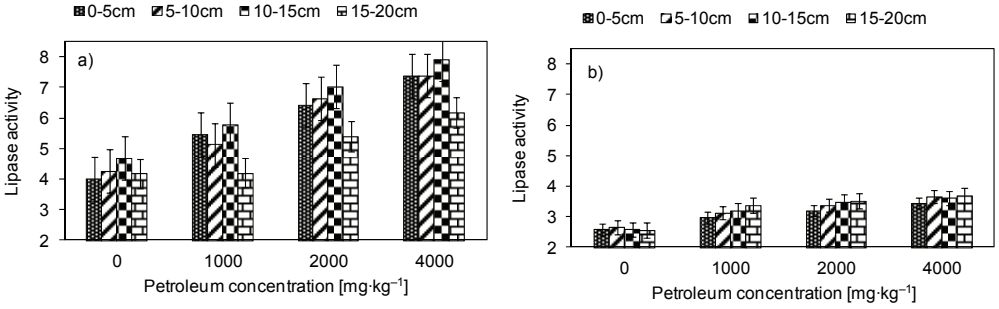

Fig. 5. Lipase activity (relative lipase units) for various petroleum concentrations: a) the group with plants, b) group without plants

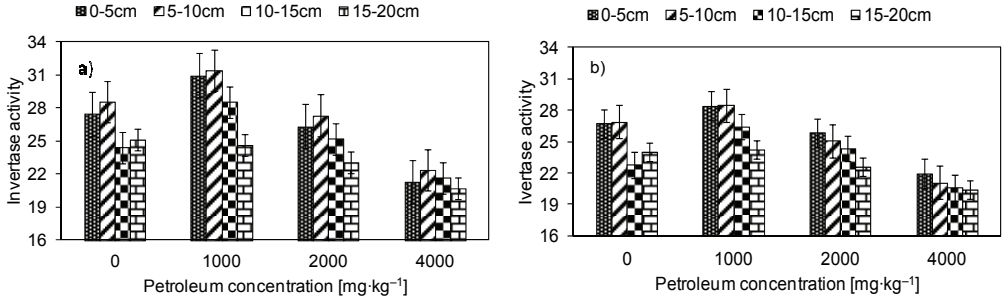

Fig. 6. Invertase activity (glucose [mg·g^{-1}· (24 h)$^{-1}$]) for various petroleum concentrations: a) the group with plants, b) group without plants

Unlike in lipase, the invertase activity (Fig. 6a, Table 3), in the group with plants, reached the maximum at the pollution level of 1000 mg·kg^{-1} and then decreased dramatically at higher pollution levels of 2000 mg·kg^{-1} and 4000 mg·kg^{-1} ($r = -0.845$, $P < 0.01$), both lower than that in the uncontaminated control. Similar phenomena could be seen in the group without plants (Fig. 6b, Table 3). Slightly higher invertase

activity in the group with plants over that in the group without plants was found at each pollution level (Fig. 1, Table 3).

Hydrolase involved in nitrogen cycle. Activities of protease and urease in the group with plants fluctuated among different petroleum pollution levels, slightly higher at the pollution level of 1000 mg·kg^{-1} and lower to a small degree at the pollution level of 2000 mg·kg^{-1} and 4000 mg·kg^{-1} compared to those in the uncontaminated control, whereas all the differences were insignificant ($P > 0.05$) (Fig. 7a, 8a), Table 3). Activities of these two enzymes were generally low in the group without plants (Fig. 7b, 8b). It is noticeable that activities of these two above-mentioned enzymes were higher in the group with plants than those in the group without plants at the same pollution level, with the differences between them reaching extremely significant level ($P < 0.01$) (Fig. 1, Table 3).

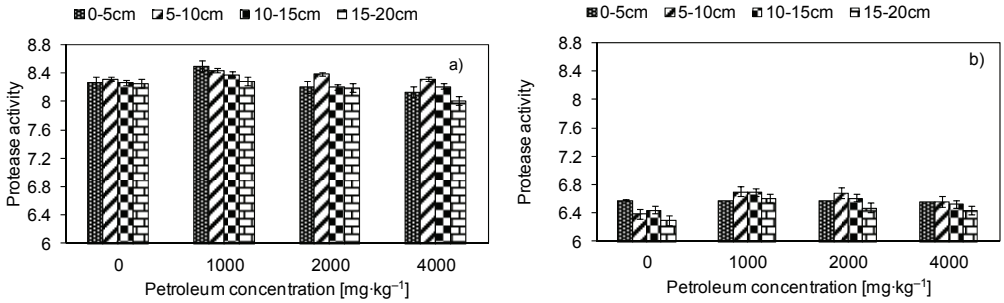

Fig. 7. Protease activity (tyrosine [mg·kg^{-1}·h^{-1}] for various petroleum concentrations: a) the group with plants, b) group without plants

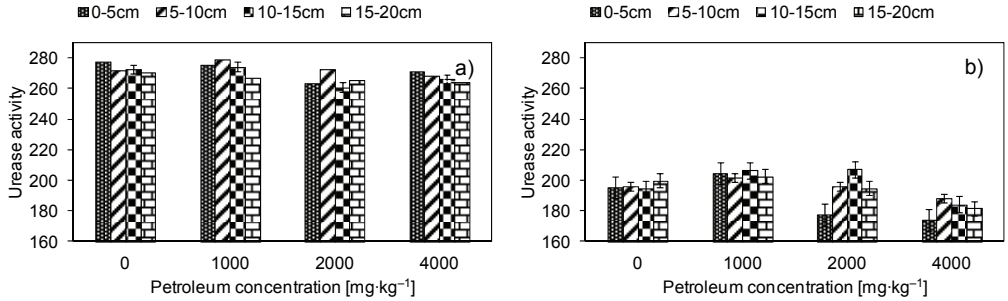

Fig. 8. Urease activity (NH_3-N [μg·g^{-1}· (24 h^{-1})]) for various petroleum concentrations: a) the group with plants, b) group without plants

Hydrolase involved in phosphorus cycle. An extremely positive correlation between activity of alkaline phosphatase and petroleum pollution concentration was

calculated both in the group with plants and in the group without plants ($r = 0.789$, $P < 0.01$ and $r = 0.963$, $P < 0.01$) (Fig. 9). Unlike in the other hydrolases, alkaline phosphatase activity in the group with plants was much lower than that in the group without plants, between which the differences were extremely significant ($P < 0.01$) at each pollution level (Fig. 1, Table 3).

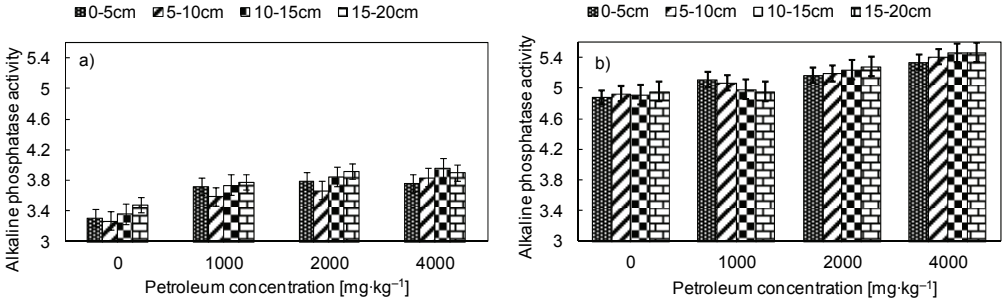

Fig. 9. Alkaline phosphatase activity (phenol [mg·g^{-1}· (24 h)$^{-1}$]) for various petroleum concentrations: a) the group with plants, b) group without plants

FDA hydrolase. FDA hydrolase, in the group with plants, displayed a higher activity at the pollution level of 1000 mg·kg^{-1}, 2000 mg·kg^{-1} and 4000 mg·kg^{-1} separately compared to that in the uncontaminated control, with its apex appearing at the pollution level of 1000 mg·kg^{-1}, reaching 187.97 μg·g^{-1}·(2 h^{-1}) (Fig. 10a, Table 3).

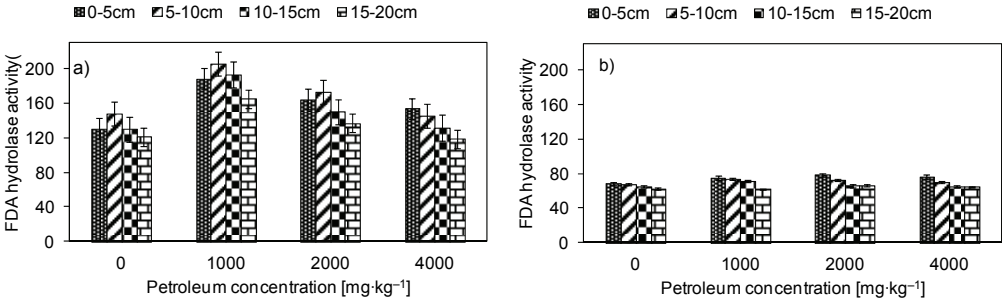

Fig. 10. FDA hydrolase activity (fluorescein [μg·g^{-1}· (2 h)$^{-1}$]) for various petroleum concentrations: a) the group with plants, b) group without plants

In the group without plants, irregular trend of the FDA hydrolase activity was observed among various pollution levels (Fig. 10b). It was obvious that FDA hydrolase activity displayed higher values in the group with plants than those in the group without plants at the same pollution level, the difference between them being extremely significant ($P < 0.01$) at each pollution level (Fig. 1, Table 3).

4. DISCUSSION

4.1. EFFECTS OF PETROLEUM POLLUTION ON THE GROWTH OF COTTON ROOTS

The data obtained for root indices suggested that cotton root grew most vigorously at petroleum pollution level of 1000 $mg \cdot kg^{-1}$, indicating a stimulating influence of low petroleum pollution on the growth of roots; contrary to this, the growth of roots was evidently inhibited at medium and high pollution levels.

4.2. SOIL ENZYMATIC ACTIVITIES AT VARIOUS PETROLEUM CONCENTRATIONS

Oxido-reductase involved in intracellular metabolism. Polyphenol oxidase, catalyzing the oxidization of phenols in aromatic compounds to quinones, is involved in the transformation of organic compounds in soil and its activity has a substantial influence on the content of phenols in soil [16]. In this study, the extremely positive correlation between the activity of polyphenol oxidase and petroleum pollution concentration, both in the group with plants and in the group without plants ($r = 0.783$, $P < 0.01$ and $r = 0.848$, $P < 0.01$) demonstrated that polyphenol oxidase activity was activated with the increment of petroleum pollution concentration. The dramatic increase of polyphenol oxidase activity in the group with plants over that in the group without plants might be contributed to the presence of cotton in the polluted soil. The boosting effect of petroleum stress on polyphenol oxidase activity indicated that polyphenol oxidase could be taken as a suitable indicator of petroleum hydrocarbon (especially phenols) pollution. The amount of polyphenol oxidase exuded from cotton roots might be enhanced by higher petroleum concentration to accelerate the degradation of aromatic pollutants in soil. The studies we have performed suggested that the presence of cotton fostered noticeably the polyphenol oxidase activity in petroleum contaminated soil, thus possibly indicating a high remediation potential of cotton. Polyphenol oxidase activity was also closely related to the content of soil nutrients. Correlation analysis made by Zhang [10] established that there was an extremely significant negative correlation ($P < 0.01$) between polyphenol oxidase activity and soil total N as well as a significant negative correlation ($P < 0.05$) between polyphenol oxidase activity and the content of organic matter and available P in soil. Hence, findings of our studies lead us to believe that the stress of high-concentration petroleum contaminants on the growth of cotton roots might be concerned with the decrease of available N and available P under such conditions.

Catalase is involved in the decomposition of hydrogen peroxide into oxygen and water through catalyzing the transfer of a pair of electrons, thereby alleviating the toxication of living organisms caused by hydrogen peroxide [30]. Catalase activity is closely related to the amount of aerobic microorganisms which constitutes one of the key factors in the microbial degradation process of petroleum contaminants [31]. Dehydrogenase, an intracellular enzyme involved in the energy transfer in the respiratory

chain, exists in almost all viable microbial cells [32]. Hydrocarbons and organic acids in soil can serve as H donors for microorganisms. Enzyme substrates, catalyzed by dehydrogenase, are oxidized by oxygen through the cytochrome system, from which process the released energy is the main energy source for heterotrophic microorganisms. In the group with plants, catalase and dehydrogenase activity was both activated at low and medium pollution level, which was shown from the increase of activities of these two enzymes at the pollution level of 1000 $mg \cdot kg^{-1}$ and 2000 $mg \cdot kg^{-1}$ compared to those in the uncontaminated control; whereas a large variance between activities of the two above-mentioned enzymes at the pollution level of 4000 $mg \cdot kg^{-1}$ could also be noticed: catalase activity was seriously inhibited while dehydrogenase exhibited, to a certain degree, tolerance against high-concentration pollution. In the group without plants, the difference of catalase activity and dehydrogenase activity among each pollution level was insignificant ($P > 0.05$), indicating that the changes of activities of these two enzymes could be mainly attributed to the alteration of soil physicochemical properties brought about by the presence of cotton roots rather than the pure variation of petroleum pollution concentration. Earlier experiments confirmed the close relationship between dehydrogenase activity and microorganic biomass in soil, which could be viewed from the standpoint that the massive growth of microorganisms leads directly to the increased activity of dehydrogenase synthesized by themselves [33]. Moreover, evidence provided by numerous studies supported the previous findings concerning a good correlation between dehydrogenase activity and petroleum degradation rate in soil [34–36]. Under condition of petroleum pollution, the proportion of the petroleum-degrading microorganism in soil would increase dramatically due to its acclimatization to petroleum stress. Based on the results of our experiment, a hypothesis was therefore advanced that the microorganic activity in polluted soil was, in general, raised owing to the presence of cotton, which was beneficial to the degradation of petroleum pollutants; the growth of oilphilic microorganisms was enhanced at low and medium petroleum pollution levels while inhibited at a high pollution level.

Hydrolase involved in the carbon cycle. Lipase, a special type of esterases widely distributed in soil, plants, animals and microorganisms, catalyzes the hydrolysis of the lipid into glycerol and fatty acids on the oil–water interface and is of great significance in soil biodynamics [16, 37]. The extremely significant positive correlations ($P < 0.01$) between soil lipase activity and petroleum pollution concentration, both in the group with plants and in the group without plants, showed that soil lipase activity was activated by petroleum contaminants. Lipase activity could also be taken as an effective indicator of the bioremediation course in freshly petroleum contaminated soil [22]. Generally, there are two pathways for the utilization of pollutants by petroleum-degrading microorganisms in soil: one is to use pollutants as the sole source of carbon and energy; the other is to combine pollutants with other organic matter for cometabolism. In our study, the increase of lipase activity might be the consequence of stimula-

tion on the growth of microorganisms brought about by oily substances contained in petroleum contaminants.

Based on the above analysis, we made the following conjecture: the increase of petroleum concentration provided a more abundant source of carbon and energy or co-metabolic substrates to foster the growth of microorganisms and simultaneously this trend was further strengthened by the amelioration effect of cotton roots on polluted soil, which was shown up by the striking changes of lipase activity among different petroleum pollution concentrations in the group with plants. The variation trend of soil lipase activity at high pollution level did not coincide with that of catalase and dehydrogenase, which might be concerned with the special biochemical functions of lipase itself in soil. As was mentioned previously, soil lipase plays an important role in soil biodynamics. At the same time, plant roots are also involved in the adsorption and transfer process of petroleum contaminants in soil. Data presented by Wang [38] gave evidence that the presence of reed roots could promote the downward migration of petroleum contaminants in soil, resulting in the vertical distributive characteristic of petroleum content in the planted soil differing from that in the unplanted soil. There is a reason therefore, based on our results, to conjecture that cotton roots might also have a boosting effect on the transfer of petroleum contaminants in order to amass pollutants around root zones, thereby favourable for the utilization and degradation of pollutants by roots and at the same time restrain the dispersion of pollutants in soil. This process might be enhanced by the increase of petroleum pollution concentration, thus activating soil lipase activity. This might also help to explain why the variation trend of soil lipase activity was not the same as that of catalase, dehydrogenase and other hydrolases at high petroleum pollution level.

Invertase was involved in the catalysis of sucrose into glucose and fructose, the products of which could serve directly or indirectly as the source of carbon for plants and microorganisms [10]. Soil invertase has correlations with many soil factors and its activity cannot only characterize the strength of soil bioactivity but also be taken as an important index for the measurement of the degree of soil maturation and the soil fertility level. Studies by Jiao [39] showed that an extremely significant positive correlation ($P < 0.01$) between soil invertase activity and soil total N, total C, total P, available P and available N existed and a conclusion was therefore made that invertase activity, under normal circumstances, was enhanced by the increase of the soil fertility level. In this experiment, invertase activity, at the pollution level of 1000 $mg \cdot kg^{-1}$ in the group with plants, was higher than that in the uncontaminated control and the growth of plant roots was also stimulated, indicating that cotton might utilize petroleum contaminants of low concentration as one of its sources of carbon to benefit the growth of itself; whereas invertase activity decreased sharply and the growth of roots was also seriously inhibited at the pollution level of 2000 $mg \cdot kg^{-1}$ and 4000 $mg \cdot kg^{-1}$, with a possible explanation being that although the total amount of organic pollutants was raised in soil of medium and high pollution concentration. The available C was

actually diminished which was detrimental to the growth and development of the plant itself. Such claims could be further supported by the evidence that the degree of inhibition on the growth of the plant was aggravated with the increase of petroleum pollution concentration. Laterally, soil invertase activity was slightly enhanced in the group with plants compared to that in the group without plants while the difference between them did not reach a significant level ($P > 0.05$), indicating that the carbon content (especially available C) as well as the overall fertility level in polluted soil was probably raised to certain extent by the presence of cotton.

Hydrolase involved in the nitrogen cycle. Soil protease specially catalyzes the conversion of protein nitrogen in organic compounds to inorganic nitrogen which could be directly absorbed and utilized by plants. Urease, an enzyme catalyzing the hydrolysis of urea into ammonia, carbon dioxide and water, is the only hydrolase that acts upon urea in soil nitrogen cycle and its activity reflects directly the N supplying power of soil [16]. Inorganic nitrogen compounds, the products of protease and urease in soil, could serve as the main source of nitrogen for plants and is closely related to the available N in soil. Protease and urease activity is also an important index for the measurement of content of nitrogen and organic matter in soil. In group with plants, the increase of protease and urease activity at the pollution level of 1000 $mg \cdot kg^{-1}$ demonstrated that the content of nitrogen and organic matter was probably enhanced by low petroleum pollution concentration, which was beneficial to the growth of plants; while the decrease of activities of the two above mentioned enzymes at the pollution level of 2000 $mg \cdot kg^{-1}$ and 4000 $mg \cdot kg^{-1}$ manifested that the content of nitrogen and organic matter probably diminished at a medium and high pollution level, which was unfavourable for the growth of plants. This was further corroborated by the variation trend of cotton roots among various pollution levels. Moreover, protease and urease, two important enzymes involved in the process of N-mineralization, have a marked influence on the available N in soil. Petroleum contaminants typically exert an adverse effect on the N-mineralization process. Based upon the above assumption, the decrease of available N in soils with medium and high concentration of pollution might be associated with the inhibition of the N-mineralization under such conditions. The noticeable increased activities of the two above-mentioned enzymes in the group with plants over those in the group without plants at the same pollution level probably demonstrated that the content of nitrogen and organic matter in soil was dramatically enhanced by the presence of cotton and the fertility level of petroleum contaminated soil was consequently raised to a large extent.

Hydrolase involved in the phosphorus cycle. Alkaline phosphatase belongs to a group of inducible enzymes responsible for the dephosphorylation of many molecules in soil and its activity is closely associated with the available P in soil [16]. A good positive correlation between soil alkaline phosphatase activity and petroleum pollution concentration was

observed. In general, alkaline phosphatase was induced by the relative low level of available P in soil. The negative correlation between alkaline phosphatase activity and the available P in various types of soil has been observed previously [40]. Our findings showed that the content of available P in polluted soil probably decreased with the increment of petroleum pollution and was raised to certain extent by the presence of cotton, which could be seen from the lower activity of alkaline phosphatase in the group with plants compared to that in the group without plants.

FDA hydrolase. FDA hydrolase belongs to a group of hydrolases including protease, lipase, esterase, etc. and its activity could serve as an appropriate indicator of the overall enzymatic activity in soil [29]. FDA hydrolase is closely related to total C, total N, total P and other nutrient indices in soil and moreover, it has a close correlation with soil microorganic activity, thus being able to reflect comprehensively the conversion status of organic matter and the overall microorganic activity in the soil environment [29, 41, 42]. In the group with plants, FDA hydrolase showed the highest activity at the pollution level of 1000 $mg \cdot kg^{-1}$, indicating that the overall enzymatic activity and the overall microorganic activity in soil was probably both enhanced by low-concentration petroleum pollution, which could be further corroborated by the fact that cotton roots grew most vigorously at this pollution level. Compared to the group without plants, a higher FDA hydrolase activity was observed in the group with plants, implying that the presence of cotton probably raised the overall enzymatic activity and the overall microorganic activity in petroleum polluted soil. What is more, a close correlation between FDA hydrolase activity and protease activity as well as between FDA hydrolase activity and invertase activity was also observed in this experiment (correlation coefficient: $r = 0.739$, $P < 0.01$ and $r = 0.727$, $P < 0.01$, respectively), indicating that protease and invertase might possess a higher sensibility in measuring the overall enzymatic activity in soil. The correlation between activities of various enzymes may also reflect the correlation between their origins [16]. The physicochemical properties of soil itself, conditions of enzymatic reactions as well as the difference of stability of various enzymes in the same medium, etc. may account for the reason why the variation trend of FDA hydrolase activity was inconsistent with that of several other enzymes in petroleum contaminated soil.

5. CONCLUSIONS

- The growth of cotton roots was boosted in low-concentration petroleum pollution (1000 $mg \cdot kg^{-1}$) at which pollution level of such root indices as the length of the main root, total root amount, root amount of a single plant and root weight of a single plant were higher compared to those at medium concentration (2000 $mg \cdot kg^{-1}$), high concentration (4000 $mg \cdot kg^{-1}$) and uncontaminated control (0 $mg \cdot kg^{-1}$); whereas the

growth of roots was inhibited at medium and high concentrations of pollution and the degree of inhibition was aggravated with the increase of pollution concentration.

- In the petroleum contaminated soil which underwent phytoremediation with cotton for 90 days, activities of polyphenol oxidase, lipase and alkaline phosphatase exhibited an extremely significant positive correlation ($P < 0.01$) with petroleum pollution concentration, implying that activities of the three above mentioned enzymes could serve as a sensitive indicator of the degree of petroleum pollution for soil; activities of catalase and dehydrogenase both reached the maximum at the pollution level of 2000 $mg \cdot kg^{-1}$; activities of invertase, protease, urease and FDA hydrolase were highest at the pollution level of 1000 $mg \cdot kg^{-1}$.
- Compared to the group without plants, activities of all enzymes except for alkaline phosphatase were higher in the group with plants at the same pollution level and most of the differences between them reached significant ($P < 0.05$) or extremely significant ($P < 0.01$) level.
- Based on the results described above, a hypothesis was advanced as below: The content of carbon, nitrogen and phosphorus in the petroleum contaminated soil was raised dramatically by the presence of cotton and the fertility level of the polluted soil was accordingly enhanced. Similarly, the overall enzymatic activity and the overall activity of indigenous microorganisms in the polluted soil were both strengthened owing to the presence of cotton, which was advantageous to the degradation of petroleum contaminants. Thus, cotton might be an appropriate plant for the remediation of the petroleum contaminated soil in North China.

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