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Stomatal Response of Maize (*Zea mays* L.) to Crude Oil Contamination in Soils

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Abstract: In this study, maize plant was cultured in soil contaminated with different levels of crude oil. The purpose was to investigate the change of soil properties, leaf physiological and chemical parameters, and phenanthrene content in the leaf. Results showed that soil water content significantly increased when the levels of total petroleum hydrocarbons were 3700–17,800 mg/kg in soil, and soil electrical conductivity significantly increased compared with the control. In maize leaf, stomatal length and density, as well as K and Na contents decreased in contaminated treatments compared with the control. Stomatal length has a significant positive correlation with K content in leaf ($r = 0.92$, $p < 0.01$), while stomatal density was negatively correlated to the crude oil level in soil ($r = -0.91$, $p < 0.05$). Accumulation of phenanthrene in maize leaf was mainly through the foliar uptake pathway. Phenanthrene concentrations of maize leaf in oil-treated soil were less than that of the control, which exhibited a significant positive relationship with stomatal length ($r = 0.98$, $p < 0.01$). This study demonstrated that the stomata structure of maize could be influenced by crude oil and thus possibly controlling the accumulation of polycyclic aromatic hydrocarbons in aerial tissues. Based on these results, controlling stomata movement will be beneficial to phytoremediation of contaminated soil.

Keywords: maize; stomata; soil; phenanthrene; remediation

1. Introduction

Petroleum oil is the main energy source and plays an important role in modern society. Soil contaminated with petroleum hydrocarbons is an increasing environmental concern as oil consumption increases dramatically around the world [1]. Among the numerous components of petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) are persistent and carcinogenic in the environment, and thus threaten human health through contaminated food chain [2]. In general, compared with inhalation or skin contact, ingestion of contaminated food is the primary pathway for human to exposure to PAHs [3,4].

Oil residuals can cause some major changes in the soil chemical properties, such as decreased total nitrogen content, and increased pH value to some extent [5], which influences the growth of plants in soil. The toxic effects of crude oil contamination prevent germination from occurring and provide unsatisfactory soil conditions [6,7]. Such poor soil conditions may result from insufficient aeration caused by decreased air-filled pore space, low water content, as well as a reduction of available nutrients [8]. Therefore, it is necessary to fully understand the changed properties of petroleum hydrocarbon contaminated soils that are closely related to plant growth.

Plants adapt to environmental stress by adjusting their external morphology, internal structure, and physiological and ecological characteristics [9]. Crude oil is likely to directly affect plants after contact and provide a resulting intake of contaminants [10,11]. Plants take in PAHs mainly through soil-to-plant and air-to-plant, i.e., root uptake and atmospheric deposition from gaseous or particulate forms [12,13]. In the former pathway, PAHs can be taken in the aerial plant tissues from the root through the transpiration stream within the xylem, while in the latter one, they can be diffused into plant leaves via the cuticle or the stomata [14]. Since stomatal closure or opening is vital to the transpiration and gas exchange, it should be considered in the study of PAHs uptake by plants.

In botany, a stoma is a pore found in the epidermis of leaves, stems, and other organs controlling gas exchange between the atmosphere and plants [15]. Stomatal density and aperture (length of stomata) vary under many environmental factors such as atmospheric CO_2 concentration, light intensity, air temperature [16,17], potassium and sodium concentration [18], air pollution, and environmental stress [19]. For example, stomatal size obviously decreases with water deficit, and stomatal density is positively correlated with stomatal conductance, net CO_2 assimilation rate, and water use efficiency [20]. Thus, the stomata can adapt to local and global changes on all time scales from minutes to millennia [15].

The edible plants grown in contaminated soils are of great concern to human health for its potential risk. For instance, maize plant has been reported to be a candidate for phytoremediation of hydrocarbon-contaminated soil [21,22]. In our previous work, maize plant was also applied for phytoremediation of crude oil contaminated soil, in which several PAHs had been detected in maize plants, and phenanthrene (PHE) had the highest level [23,24]. Stomata play an important role in the air-to-plant uptake pathway of PAHs. However, the information about the changes in leaf stomata of maize plants grown in crude oil contaminated soils is scarce. Therefore, it is necessary to investigate the changes of leaf stomata of plants grown in contaminated soil and the influence factors. The main objectives of this study were (1) to understand the changes of stomata structure and the concentrations of potassium (K) and sodium (Na) in maize leaf in response to crude oil contaminated soil; (2) to evaluate the relationships between the PHE (a representative of PAHs) leaf concentration and leaf parameters/soil properties.

2. Materials and Methods

2.1. Chemicals, Seed of Maize and Soil

Crude oil without refining was obtained from Guangzhou Department, Sinopec Corporation, China. All other agents used in this study were analytical grade. Seed of CT 38 was purchased from Research Institution of Crop, Guangdong Academy of Agricultural Sciences, China.

The soil in the experiment was collected from the upper layer (0–20 cm) of an abandoned farm in Guangzhou Higher Education Mega Center, Guangzhou, China. After stones and roots were removed, the soil was air-dried, smashed, and passed through a 4 mm sieve. The organic matter content and pH of the soil were 1.3% and 6.54, respectively. Nutrient levels were 24.5 g/kg ammoniac nitrogen, 4.32 g/kg total phosphorus (P), and 0.40 g/kg total K.

2.2. Experimental Design and Management

The soil (1.5 kg) was placed in a plastic crate, spiked with different amounts of crude oil, and stirred for homogeneity with a wood spoon. The soil was then put into plastic pots and placed outdoors for 4 months to adequately evaporate the volatile fractions of crude oil. And then the total petroleum hydrocarbon (TPH) levels were measured to be 0, 2600, 3700, 6500, 17,800, and 48,800 mg/kg, respectively, using the method of our previous work [23]. Each treatment was replicated three times. Three maize seeds were placed into soils at 2 cm depth in each pot. After the maize seedlings grow out of the soils with three expanded leaves, one seedling was left in each pot. The pots were placed outdoors at the top of our laboratory building. The experiment was started in September. The average

temperature was 23.2 °C during the experiment. Water was added into potholders for soil moisture. After two months, soil properties and maize leaf parameters were determined.

2.3. Analytical Methods

2.3.1. Soil Water Content and Soil Electrical Conductivity

To understand the water content of soil contaminated with different levels of crude oil, soil samples were collected in pots with a core sampler when water sufficiently infiltrated into the soil from a potholder. Soil water content was determined gravimetrically by weighing, after drying in an oven at 105 °C for 12 h according to the method described by Liu et al. [25]. Soil electrical conductivity was measured by a portable electrical conductivity meter (Hanna HI-993310D).

2.3.2. Determination of Stomatal Traits

Stomatal traits in maize leaf were determined according to the method described by Zheng et al. [26]. The first leaf fully expanded on the main stem was sampled for each plant. Colorless nail polish was carefully smeared on leaf samples for about half an hour. Then the thin film was immediately covered with a cover slip and pressured lightly with a fine-point tweezer. Leaf stomatal length and density were measured from the base, middle, and tip sections on leaves of maize. Three slides were prepared for each taxon. Stomatal length was determined by micro-morphological observations carried out on 1 cm^2 portion per leaf (excised from similar areas) with a microscope (Carl Zeiss Micro-imaging, GER) equipped with a spot insight color camera (Diagnostic Instruments, Sterling Heights, Sterling Heights, MI, USA). Stomatal density (NO/cm^2) was calculated on 10 representative fields of leaves according to the method described by Orsini et al. [27].

The gravimetric measurement of water loss after leaf excision is a rapid method to evaluate the transpiration rate. The initial fresh weight (FW) and the weight after 5 min (W) were recorded. Water loss in 5 min was the difference between FW and W, which were used to calculate the transpiration rate [28]. The leaf water content was also determined gravimetrically by the method of soil water content mentioned above. The leaves were cut into pieces and dried in an oven at 105 °C until they reached a constant weight.

2.3.3. Determination of K and Na Concentration in Maize Leaves

To determine the concentration of K and Na in maize leaves, samples were collected and dried, followed by digestion with HNO_3 and oxidation by H_2O_2 with a heating plate. The residual was dissolved in 5% (V/V) HNO_3 solution, the concentrations were measured by atomic absorption spectrophotometry (AAS, Z-2000, Hitachi, Tokyo, Japan) as previously described by Cicek and Cakirlar [29].

2.3.4. Determination of PHE in Maize Leaves

Concentration determination of PHE in maize leaf was conducted according to the previous method described by Tao et al. [30] with some modification. Maize samples (1.00 g) homogenized with about 1 g of anhydrous sodium sulfate were put in glass tubes. The samples were extracted with 10 mL hexane/dichloromethane (1:1) under ultrasonic conditions for 30 min. Then the extract was collected in a beaker. This process was replicated three times. The collected extract was purified by passage through a silica gel column and vacuum concentrated with a rotary evaporator at 40 °C. The samples were re-suspended in *n*-hexane to a final volume of 1 mL for further analysis by gas chromatography mass spectrometry (GC-MS).

Analysis of plant samples was conducted using a GC–MS with Thermo Trace GC Ultra instrument coupled to a Thermo DSQ II mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). Compounds were separated in a 30 m 0.25 mm id capillary column coated with 0.25 µm film (HP-5MS, Agilent, USA). GC temperature was programmed from an initial 80 °C before commencing at 10 °C/min

up to 290 °C, with a final holding time of 10 min. Helium was used as carrier gas. A 1.0 µL aliquot of the extract was injected while the injector port was held at 280 °C and operated in a splitless mode at a flow rate of 1.0 mL/min. The head column pressure was 30 kPa. The mass spectrometer was operated in scan mode with an electron impact ionization of 70 eV and an ion source temperature of 230 °C. Solvent delay was set at 4 min. Selective ion monitoring model was used. The target ions and retention time was 178 and 14.84 min for PHE, respectively [31].

2.4. Statistical Analysis

Statistical Product and Service Solutions statistic software 17.0 (SPSS company, Chicago, IL, USA) was used for the statistical evaluation of the results designed as completely randomized with three replicates of each parameter. Mean values followed by the same letter were not significantly different, as determined by an analysis of variance (ANOVA). The differences were compared by Duncan's range at a significance level of $p < 0.05$. The relationships between parameters were evaluated by Pearson correlation analysis.

3. Results

3.1. Changes in Soil Properties

The changes in soil water content and soil electrical conductivity in different treatments were shown in Figure 1. Soil water content was significantly increased when the TPH levels rose from 3700 to 17,800 mg/kg, but dramatically decreased at the extremely high level of 48,800 mg/kg, compared to the control soil (Figure 1A). At the low-level contaminated soil (2600 mg/kg), water content was similar to that of the control. The values of soil electrical conductivity in the contaminated treatments were significantly higher than that of the control (Figure 1B), but it exhibited no regular tendency.

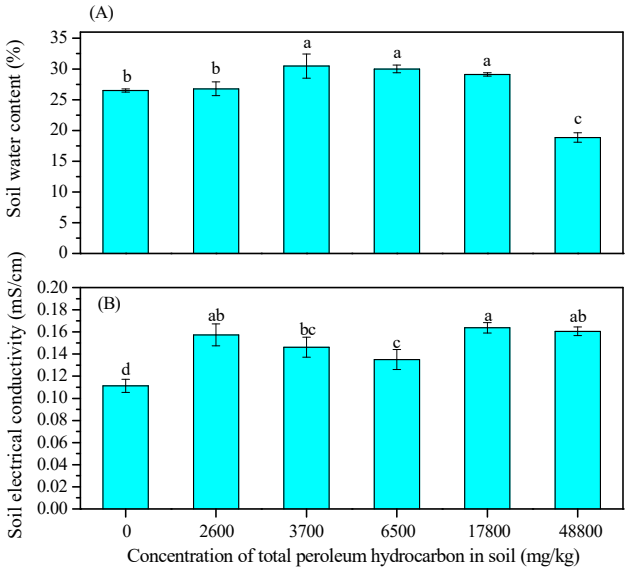

Figure 1. Effect of soil contamination with crude oil on soil water content (**A**) and soil electrical conductivity (**B**). ($p < 0.05$). Different letters on top of the bar indicate they are significantly different at $p < 0.05$.

3.2. Leaf Growth and Stomatal Density and Length

Stomatal length of maize leaf in contaminated treatments significantly decreased in comparison with that in the control (Figure 2A), but there were no significant differences among 3700–48,800 mg/kg treatments. Stomatal density decreased with increasing TPH levels in soil (Figure 2B). In the highest-level contaminated soil, stomatal density was decreased by 46% compared with the control. In comparison,

the downtrend showed that stomatal length was more sensitive than stomatal density to contaminated soil.

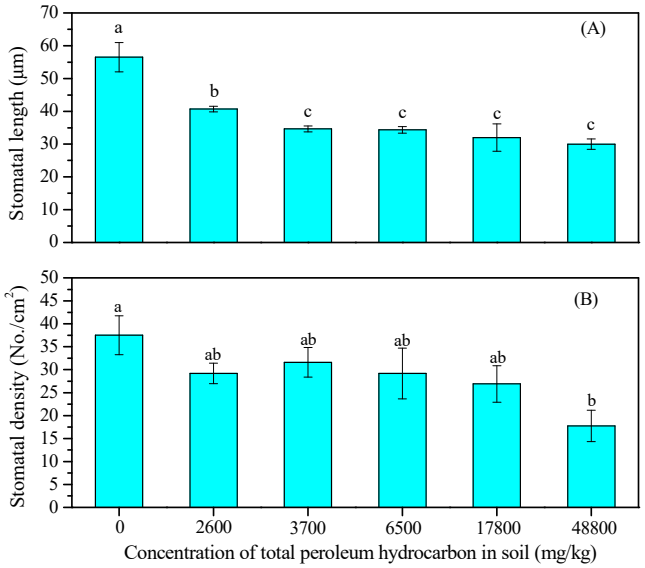

Figure 2. Effect of soil contamination with crude oil on stomatal length (**A**) and stomatal density (**B**) of maize leaf. ($p < 0.05$). Different letters on top of the bar indicate they are significantly different at $p < 0.05$.

3.3. Water Content and Transpiration Rate

Water content and transpiration rate are important physiological functions of plants, which may be influenced by soil conditions. As shown in Figure 3A, leaf water contents in all samples grown in contaminated soil were similar, suggesting the crude oil contaminated soil with different concentration did not have a remarkable effect on the water transport from soil to plant tissues, but did change the water content in the maize leaf. As shown in Figure 3B, transpiration rates of maize leaf in contaminated soil did not exhibit a significant difference, but were slightly higher than the control. This indicated the transpiration rate of maize plant could be affected by crude oil contaminated soil to some extent.

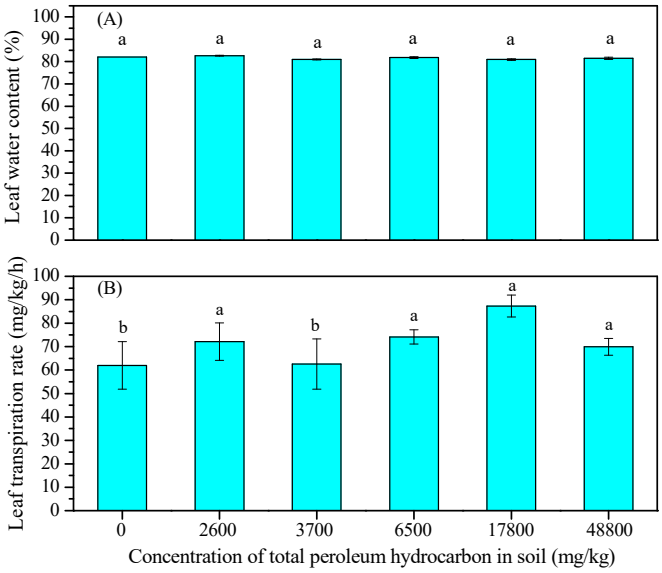

Figure 3. Effect of soil contamination with crude oil on water content (**A**) transpiration rate (**B**) of maize leaf ($p < 0.05$). Different letters on top of the bar indicate they are significantly different at $p < 0.05$.

3.4. Concentrations of K and Na in Maize Leaf

As the major mineral elements in plant tissues, K and Na play important roles in maintaining the physiological functions, especially in regulating the opening and closure of stomata. It is necessary to investigate the effect of crude oil contaminated soil on the K and Na assimilation of maize. As shown in Figure 4, both K and Na concentrations in maize leaf significantly decreased with increasing TPH levels of soils, indicating the crude oil contaminated soil had a significant effect on the assimilation of K and Na in maize plant. Additionally, K and Na concentrations in maize leaf at high levels of contaminated soil (above 6500 mg/kg) were much lower than those of the control.

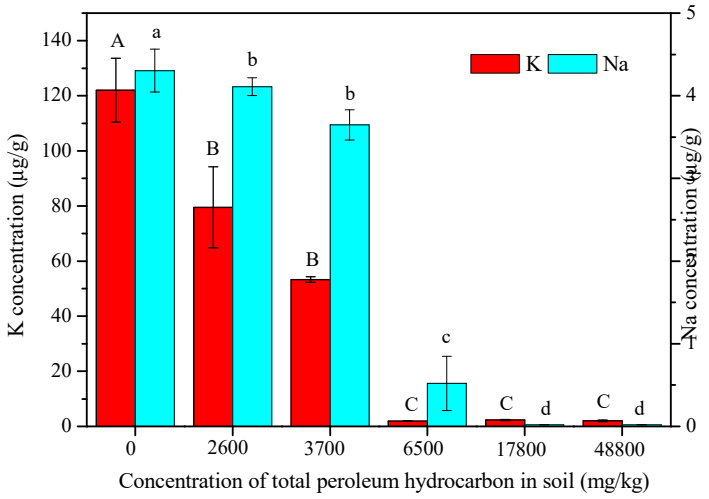

Figure 4. Effect of soil contamination with crude oil on K and Na concentrations in maize leaf. ($p < 0.05$). Different letters on top of the bar indicate they are significantly different at $p < 0.05$.

3.5. Phenanthrene Concentration in Maize Leaf

As shown in Figure 5, PHE concentrations of maize leaf in contaminated treatments were lower than that in the control group, and there were significant differences when TPH levels reached 3700 mg/kg. Furthermore, in the soil treatments with TPH levels ranging from 3700 to 48,800 mg/kg, PHE concentrations in maize did not exhibit significant changes.

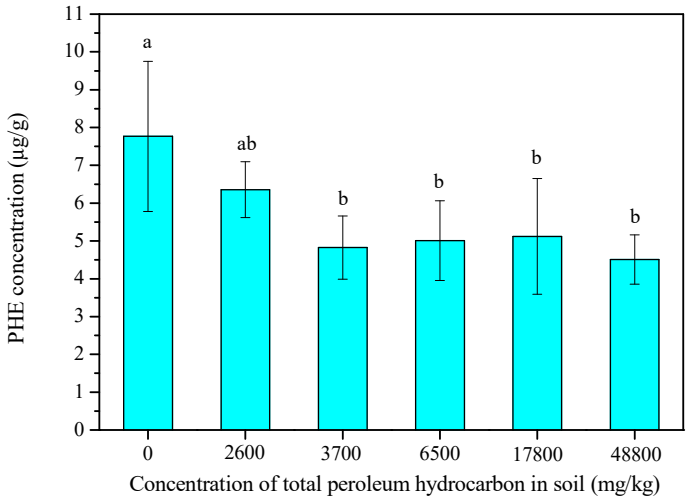

Figure 5. Phenanthrene concentration of maize leaf in different treatments. ($p < 0.05$). Different letters on top of the bar indicate they are significantly different at $p < 0.05$.

3.6. Relationship

The relationships between different parameters were presented in Table 1. TPH level in soil was negatively correlated to soil water content, leaf water content, stomatal length and density, K, Na and PHE contents in maize leaf, but positively correlated to soil electrical conductivity and transpiration rate of maize leaf. Especially, significant negative correlations were observed between soil TPH level and soil water content ($r = -0.82$, $p < 0.05$), as well as stomatal density ($r = -0.91$, $p < 0.05$). In this study, correlation analysis also covered the stomata structure, ion contents, and PHE concentration in maize leaf. Stomatal length was significantly positively correlated to leaf K content ($r = 0.92$, $p < 0.01$). Besides, PHE concentration had a significantly positive correlation with stomatal length ($r = 0.98$, $p < 0.01$).

Table 1. Simple correlation coefficient (r) between parameters.

	TPH	SW	EC	LW	TR	SL	SD	K	Na	PHE
TPH	1.00									
SW	−0.82 *	1.00								
EC	0.55	−0.22	1.00							
LW	−0.10	−0.25	0.23	1.00						
TR	0.27	0.10	0.65	−0.28	1.00					
SL	−0.59	0.10	−0.83 *	0.05	−0.55	1.00				
SD	−0.91 *	0.65	−0.78	−0.10	−0.46	0.81	1.00			
K	−0.62	0.11	−0.66	0.30	−0.67	0.92 **	0.79	1.00		
Na	−0.71	0.27	−0.54	0.44	−0.72	0.75	0.78	0.94 **	1.00	
PHE	−0.59	0.09	−0.71	0.11	−0.40	0.98 **	0.76	0.89 *	0.72	1.00

TPH: total petroleum hydrocarbon in soil; SW: soil water content; EC: soil electrical conductivity; LW: leaf water content; TR: transpiration rate; SL: stomatal length; SD: stomatal density; K: leaf K content; Na: leaf Na content; PHE: phenanthrene concentration in leaf. * indicate the r values are significant at $p < 0.05$. ** indicate the r values are significant at $p < 0.01$.

4. Discussion

4.1. Soil Properties in Crude Oil Contaminated Soil

Soil properties play an important role in soil microorganism activity and plant growth. According to previous work, plants exposed to soils contaminated with petroleum hydrocarbons were subjected to growth limitations, due to low water uptake and reduced nutrient availability [8]. Mineral nutrient availability can be reflected by soil electrical conductivity. Therefore, soil water content and soil electrical conductivity need to be well understood in phytoremediation of soil contaminated with petroleum hydrocarbons. In this study, water contents increased in soil contaminated with certain TPH levels (3700–17,800 mg/kg) but decreased significantly when TPH levels reached 48,800 mg/kg. Water-stable aggregates in soil are related to the content of soil organic matter [32]. The addition of crude oil to soil increased the soil organic matter content, possibly resulting in enhanced water holding capacity at a certain limited range, as soil organic matter was an important determinant of the available water capacity [33]. However, the high concentration of crude oil in soil might prevent water from entering the pores of soil, which decreases water holding capacity.

Soil electrical conductivity increased in crude oil contaminated soils compared to the control soil in this study. This result was in agreement with previous work, which showed that the value of electrical conductivity in contaminated soil was higher than that of the control site [5]. Soil electrical conductivity represents soil salinity, which is mainly composed of cation ions such as Na^+, K^+, and Ca^{2+}. The result of this study illustrated that availability of these ions in soil was not the limiting factors for plant uptake. The addition of crude oil in soil leads to higher soil electrical conductivity, possibly resulting from the production of metabolites from crude oil biodegradation [34].

4.2. Stomata and Other Leaf Parameters

Stomata are the pores in the epidermis of botany leaf controlling gas exchange, mainly CO_2 and water vapor, between the atmosphere and plants [15]. In the present work, stomatal length and density of maize leaf in contaminated soil treatments decreased compared with those in the control, indicating that crude oil contaminated soil harmed stomatal structures. And the downtrend showed stomatal length to be more sensitive than stomatal density to contaminated soil. Previous work showed stomata in plants were not only influenced by air pollution of automobile emissions [35], but also by soil or water contamination of environmental stress [19,36]. It is interesting to observe the changes in maize stomata induced by the contamination of air or soil in the present work.

Plasticity of stomatal development may be determined by many exogenous and environmental cues, of which abscisic acid (ABA) is considered as a vital regulator of environmentally determined stomatal development [37]. According to previous work, ABA can increase progressively in the root with responses to abiotic environmental stress [38,39]. In particular, the level of endogenous ABA significantly increased in pea (*Pisum sativum* L.) plant with increasing fluoranthene concentrations [40]. Therefore, crude oil contaminated soil might stimulate the synthesis of ABA in maize root and then increase the amount of ABA, thus decreased stomatal length and density.

In addition, the changed stomata might affect other leaf parameters. Stomatal length has an extremely positive correlation with leaf K content ($r = 0.92$, $p < 0.01$), and positive correlation with leaf Na content ($r = 0.75$, $p < 0.01$). It seems that K and Na content in leaf may be influenced by the stomata structure. But on the other hand, K^+ is considered to involve in controlling stomatal movements, in which guard cell K^+ uptake from the apoplast is mediated by a proton-extruding adenosine triphosphatase on the plasmalemma [41]. Moreover, tonoplast-localized NHX proteins as Na^+, K^+/H^+ antiporters are essential for active K^+ uptake at the tonoplast for stomatal function [42], so that K^+ and Na^+ in plant are considered as twins [43]. The contents of K and Na in maize leaf might also affect the length and density of stomata. The relation between those ions and stomata structure still needs further study. Additionally, leaf water content and transpiration rate did not significantly change in different treatments, indicating that the response of water balance in maize plant was different from nutrient ions in contaminated soil.

4.3. PHE Uptake and Stomata

Accumulation of PAHs in aerial plant tissues may be from root through transpiration stream and from diffusion via leaf stomata [14]. In the present work, transpiration rates in all plants were similar (Figure 3B). Besides, PHE concentrations of maize leaf in contaminated treatments were lower than that of the control. PHE could volatilize from contaminated soil. These results indicated PHE accumulation in maize aerial tissues might be from foliar uptake pathway which was controlled by stomata. This suggestion was also confirmed by the fact that PHE concentration in maize leaf was significantly positively correlated with stomatal length ($r = 0.98$, $p < 0.01$). Besides, previous studies also confirmed that foliar uptake was the dominant pathway of PHE accumulation by plant [44–46].

Since stomata play an important role in uptaking pollutants by plants grown in contaminated soils, measurements influencing stomatal movement can be applied in phytoremediation of contaminated soil for different purposes. For example, ABA can be used on maize husk for inhibition of PAH accumulation by grain due to being able to induce stomatal closure and inhibit a light-induced stomatal opening [47], when maize plant is considered for phytoremediation of PAHs-contaminated soil. Thus, safe food will be produced. In contrast, since fusicoccin can prevent dark-induced stomatal closure [48], it can be used on hyperaccumulators for extracting more pollutants in remediation of soil contaminated with heavy metals, which are taken up by plant root and transferred to aboveground tissues with a transpiration stream that closely relates to a stomatal opening. Therefore, phytoremediation of soils contaminated with organic pollutants or heavy metals can benefit from the controlling of stomatal movement. However, the data for the effect of phytohormone on maize plant was not provided in this study, which needs to be investigated in future work.

5. Conclusions

Stomatal response and the change of related parameters of the maize plant (*Zea mays* L.) to crude oil contaminated soil were investigated in this study. Soil water content and electrical conductivity increased to a certain extent in contaminated soil, whereas the TPH level exhibited a negative relationship with soil water content ($r = -0.82$, $p < 0.05$). Stomatal length and density, leaf K, and Na contents decreased in contaminated soil compared with that of the control group. Stomatal length is positively correlated to leaf K content ($r = 0.92$, $p < 0.01$), while stomatal density is negatively correlated to soil TPH level ($r = -0.91$, $p < 0.05$). Moreover, it is found that the accumulation of PAHs in maize mainly occurred through the foliar uptake pathway. And PHE concentration exhibits a significantly positive relationship with stomatal length ($r = 0.98$, $p < 0.01$). Based on this study, measurements should be applied to control stoma closure or opening for different purposes in phytoremediation of contaminated soils.

Author Contributions: C.L. and C.Z. conceived and designed the experiments; C.Z. and H.H. performed the experiments; H.H., Y.Z. and T.X. analyzed the data; C.L. and C.Z. written original draft; H.L. and H.H. reviewed and edited the draft.

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Conflicts of Interest: The authors declare no conflict of interest.

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