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PHYTOTOXITY OF POLYCYCLIC AROMATIC HYDROCARBONS TO *SALIX VIMINALIS* L.

XIA LI[1*], JUNXIANG LIU[2], FENGZHEN CHEN[1], YUNHE CHENG[3], YUANCHENG WANG[2], AO LI[2], FEIFEI ZHAI[4] AND ZHENYUAN SUN[2]

[1]*College of Agriculture and Bioengineering, Heze University, Heze 274000, Shandong, China*
[2]*Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, China*
[3]*Beijing Academy of Forestry and Pomology Sciences, Beijing 100093, China*
[4]*School of Architectural and Artistic Design, Henan Polytechnic University, Jiaozuo 454000, Henan, China*
[*]*Corresponding author's email: 393362284@qq.com*

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are toxic, mutagenic, and carcinogenic, causing serious risks to human health and ecosystem security. High concentrations of pollutants can cause growth inhibition and even survival stress to plants. This work aimed to explore the phytotoxicity of PAHs to *Salix viminalis*. In this study, cut seedlings of *S. viminalis* were subjected to hydroponic experiments under phenanthrene stress to examine changes in gene expression and physiology of willow roots. Under phenanthrene stress, the superoxide anion radical generation rate ($O_2^{\bullet-}$) and malonaldehyde (MDA) content significantly increased. Catalase (CAT) played major role in the detoxification of reactive oxygen species (ROS) in willow roots. Phenanthrene caused osmosis stress in roots, as shown by the increase in proline and soluble sugar content, sucrose synthase SUS3, phosphosucrose synthase SPS4, the sucrose transporters SWEET16 and SUC2, delta-1-pyrroline-5-carboxylate synthetase P5CS, rolyl 4-hydroxylase *P4H1*. Genes in root cellulose synthesis, some mineral elements transporters and auxin transporters were downregulated, which were adverse to root growth of willow. This study confirmed the toxic pathway of PAHs to willow and would provide a basis for the study of enhancing plant resistance to PAHs and strengthening the application of phytoremediation.

Key words: Gene expression; PAH stress; Physiological change; Phytotoxicity.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) produced by petroleum hydrocarbon combustion (Ball & Truskewycz, 2013) are mainly released in the environment through anthropogenic activities (industry, transportation, wastewater and sludge). PAHs are toxic, carcinogenic, and mutagenic and present serious risks to ecosystem security and human health. The removal of PAHs from the environment has become a research hotspot (He *et al*., 2015; Liu *et al*., 2015). Phytoremediation, which mainly uses plant extraction, rhizosphere degradation and cooperation with rhizosphere microorganisms to remove pollutants, is widely recognized due to its low operating cost and pollution-free nature (Tejeda-Agredano *et al*., 2013; Shahsavari *et al*., 2015). A series of studies have demonstrated the more active dissipation of hydrocarbons in soil in view of plants (Rezek *et al*., 2008; Soleimani *et al*., 2010; Wang *et al*., 2012; Shahsavari *et al*., 2015).

PAH resistance is the premise of phytoremediation by plants. At low concentrations, PAHs can be a carbon and energy source for microorganisms that degrade these compounds, whereas high concentrations of pollutants can cause growth inhibition (Petrová *et al*., 2017) and even survival stress (Alkio *et al*., 2005; Salehi-lisar *et al*., 2015), which is not conducive to phytoremediation. Fluorene treatment at 50 $mg \cdot kg^{-1}$ and 100 $mg \cdot kg^{-1}$ reduced the fresh weight, dry weight, and plant height of potted *Triticum aestivum*, *Medicago sativa*, and *Helianthus annuus* (Salehi-Lisar *et al*., 2015). Negative effects were observed on plant height and shoot and root dry weight in *Medicago sativa* and *Brassica napus* after 90 days in 100 $mg \cdot kg^{-1}$ pyrene-contaminated soil (D'Orazio *et al*., 2012). These researches had confirmed that high concentrations of PAHs generally had toxic effects on plant growth and development.

There are few reports on the toxic mechanism of PAHs in plants. Phenanthrene directly inhibits photosynthetic electron transport and rubisco carboxylation activity to decrease net Pn (Jin *et al*., 2017). Phenanthrene can also cause the cell structures of wheat leaves to become plasmolyzed and distorted, organelles to disappear, and more H_2O_2 to be produced, resulting in cell death (Shen *et al*., 2018). Plant roots are directly damaged in PAH-contaminated soil or water. Alkio *et al*., (2005) found that the expression of the cell wall-loosening protein expansin was inhibited in *Arabidopsis thaliana* in response to phenanthrene treatment. Through microRNA sequencing, Li *et al*., (2017) concluded that miR156 and miR164 directly regulate the growth and development of wheat roots by targeting SPL and NAC, respectively, and that miR398 targets CSD1 and CSD2 and miR1121 targets CAT1 to regulate oxidative reactions to respond to phenanthrene stress. Based on previous studies, we found that the phytotoxicity of PAHs is still focused on herbaceous plants and that the toxic pathways to woody plant roots need to be further studied.

S. viminalis effectively decontaminates soils polluted by PAHs. The degradation rate of anthracene, fluoranthene, and pyrene and benzo (a) pyrene in creosote soil is more than twice as high as that in unplanted soil (Önneby, 2005). A greenhouse experiment also confirmed that the degradation rates of phenanthrene and pyrene in *S. viminalis* planting soil are 1.47 and 1.27 times higher, respectively, than those without plants (Hultgren *et al*., 2009). However, the molecular

mechanism of PAH toxicity to *S. viminalis* remains unclear. Studies have suggested that the expression of genes involved in detoxification and regulation of macromolecular changes in Arabidopsis thaliana after 8 h under phenanthrene stress (Amrani *et al*., 2015) reached an expression peak at 24 h (Lin *et al*., 2016). Therefore, in this study, transcriptome sequencing was conducted on the roots of *S. viminalis* after 24 and 36 h of exposure to phenanthrene through a hydroponic test. Physiological changes with reactive oxygen species (ROS), malondialdehyde (MDA) content and osmotic regulating substances were measured in the roots of willow for 16 days after phenanthrene stress to confirm the damage caused by PAHs. This research aimed to provide a new and deeper understanding of the toxic mechanism of PAHs in plants. This work lays the foundation for further resistance breeding and strengthening of the phytoremediation strategy.

Material and Methods

Chemicals: We purchased phenanthrene from Sigma-Aldrich with a purity > 98% and used it as the model PAH. The solubility of phenanthrene in pure water was 7.3 $\mu mol \cdot L^{-1}$ at 25°C.

Plant cultivation: Woody cuttings (diameter 1 ± 0.2 cm) were got from the same clone of *S. viminalis*. After two weeks of hydroponics, the plants were transferred to modified ½ Hoagland's nutrient solution (Chen, 2017). The nutrient solution was changed every 3 days for another 8 weeks. When the height of the willow was 44–48 cm, a stress experiment was carried out in the research greenhouse under natural light.

Experimental design: Plants of uniform growth were transferred to glass containers and incubated with modified ½ Hoagland's nutrient solution, prepared with distilled water, and acclimated for 1 week. According to the response of willow to different concentrations of phenanthrene by screening test, growth was inhibited when exposed to 1.0 $mg \cdot L^{-1}$ phenanthrene. *S. viminalis* was separated to a control group and a treatment group, with three replicates in each group. The control group was modified ½ Hoagland's nutrient solution prepared with distilled water, while the treatment group was prepared with 1.0 $mg \cdot L^{-1}$ phenanthrene solution (modified ½ Hoagland's nutrient solution prepared with phenanthrene and distilled water). The pH of both groups was 6.0. The outer wall and cover plate of the container were wrapped with tin foil to keep the root system and solution away from light and to prevent photodegradation of phenanthrene. Oxygen was supplied to the culture medium throughout the process under natural light. Under phenanthrene stress for 24 and 36 h, the roots were obtained, and stored at −80°C for transcriptome determination. The roots were also collected at 4, 8, 12, and 16 days under phenanthrene stress for physiological index detection.

The determination of $O_2^{\bullet-}$ production rate, H_2O_2 and MDA content: $O_2^{\bullet-}$ production rate in willow roost was measured by hydroxylamine hydrochloride method referring to Elstner & Heupel (1976) with modifications. The H_2O_2 content was measured following Brennan & Frenkel (1977) by monitoring the absorbance of titanium peroxide at 415 nm and calculated using a standard graph. The content of MDA was determined by thiobarbituric acid method (Castrejón & Yatsimirsky, 1997; Hodges *et al*., 1999).

Enzyme extraction and assays: Superoxide dismutase (SOD) activity was determined by measuring the ability to restrain the photochemical reduction of nitroblue tetrazolium (Giannopolitis & Ries, 1977). Peroxidase (POD) activity was measured at 470 nm, referring to a previous method (Maehly & Chance, 1954). Catalase (CAT) activity was determined following Beers & Sizer (1952). The reaction mixture comprised enzyme extract, 0.05% guaiacol, 10 mM H_2O_2, and 25 mM NaP buffer (pH 7.0). The results were confirmed by determining the change in the production of dioxygen at 240 nm in 1 min using the supernatant with 90 mM NaP buffer (pH 7.0) containing 18 mM H_2O_2.

The determination of osmotic regulating substance content: The proline content was determined following the acid ninhydrin method (Vieira *et al*., 2010), with modifications. The soluble sugar content was measured using the anthrone-colorimetric method (Fan *et al*., 2004), with modifications. The soluble protein content was determined using the coomassie brilliant blue method (Tao *et al*., 2018).

Transcriptome sequencing: This work was completed by Guangzhou Genedenovo Biotechnology Co., Ltd. (China). Total RNA extraction, sequencing and analysis method were described in supplementary file.

Statistical analysis

The data of physiological index were mapped and analyzed by GraphPad Prism Version7.0 (GraphPad software, La Jolla, CA, USA). Univariate analysis of variance and multiple comparison (Tukey test) were performed.

Results

Effect of PAH stress on ROS and MDA content of willow roots: Abiotic stress conditions lead to physiological and biochemical changes, usually resulting in the accumulation of ROS. These substances include the $O_2^{\bullet-}$ hydroxyl radical (OH·), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). It is considered an important factor inhibiting plant growth and development (Mittler, 2017). The root $O_2^{\bullet-}$ production rate increased significantly under phenanthrene treatment, and the highest value was 2.21 times that of the control at day 4, and then decreased, but it was continuously higher than that of the control ($p<0.05$; Fig. 1). The H_2O_2 content was significantly higher than that of the control group only on day 4 ($p<0.05$), then decreased rapidly, and was lower than that of the control group from day 8 to 16 ($p<0.01$). The MDA content in the roots was always higher than that of the control and increased the fastest on the 8th day, with 1.24 times the control under phenanthrene stress.

Effect of PAH stress on antioxidase activity of willow roots: There are several reports of antioxidant responses to PAH stress of plants (Daresta *et al*., 2015; Shen *et al*., 2016, 2018). As shown in Fig. 2, SOD and CAT activity of roots were mostly higher than that in the control at 4 days and then decreased with prolonged stress time. POD activity was not decreased, but it was not significantly different from that in the control group throughout most of the experiment. Although CAT activity declined after 4 days, it was always higher than that in the control throughout the experiment.

Effect of PAH stress on osmotic regulatory substances of willow roots: Osmotic regulatory substances are markers of plants under water stress. The proline content in roots was elevated from 8 to 16 days ($p<0.01$, Fig. 3), and the content was 1.44, 1.12, and 1.20 times that of the control. The soluble sugar content of the roots increased significantly on days 4 and 8 ($p<0.01$), and was 1.53 and 1.63 times that of the control group, respectively. The soluble sugar content decreased in the treatment group at 12 and 16 days and was not significantly different from the control group. There was no significant difference in soluble protein content between the treatment and control throughout the experiment ($p>0.05$).

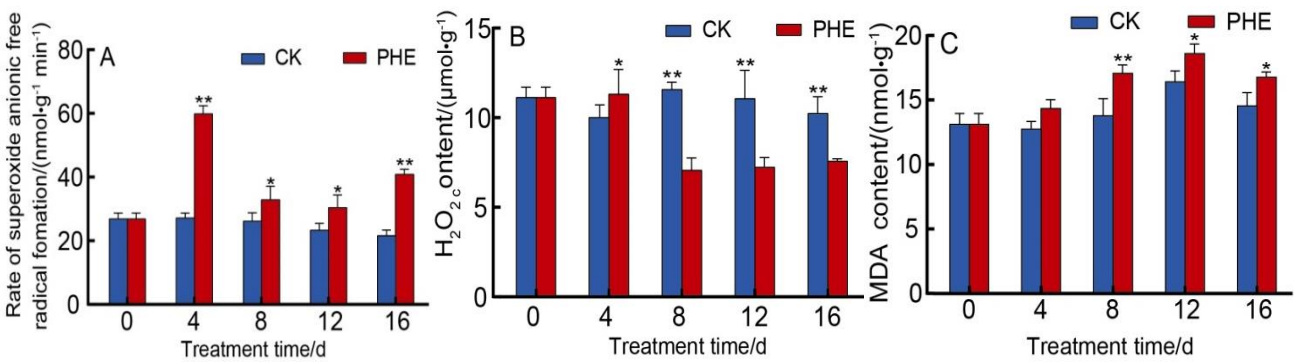

Fig. 1 Changes in $O_2^{\cdot-}$ (A), H_2O_2 (B), and MDA (C) content in the roots of *S. viminalis* of the control (CK, willow cultivated with modified ½ Hoagland's nutrient solution prepared with distilled water) and phenanthrene treatment groups (PHE, willow cultivated modified ½ Hoagland's nutrient solution prepared with phenanthrene and distilled water). Data presented are means (±SD), n=3 replications for control and treatment. A one-way ANOVA was applied with the control and phenanthrene treatment group, and $^{**}p<0.01$, $^{*}p<0.05$.

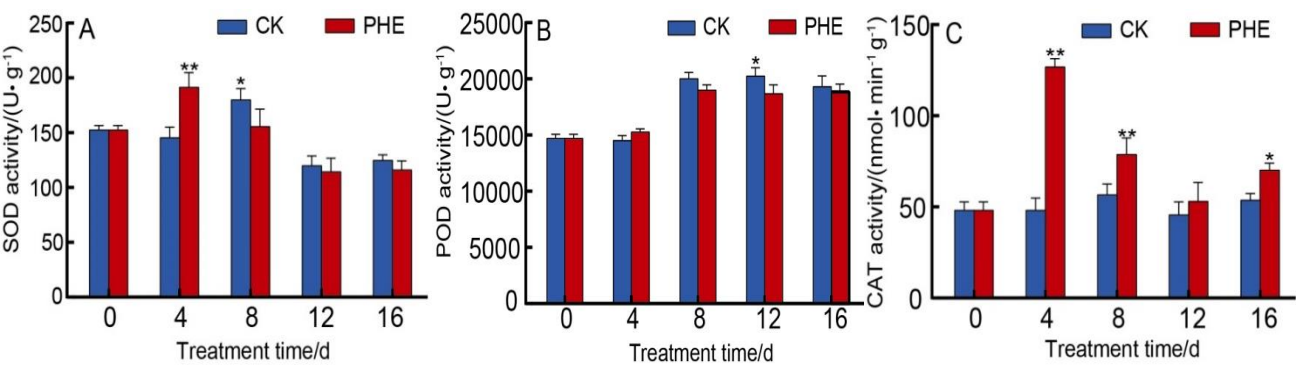

Fig. 2. Changes in SOD (A), POD (B), and CAT (C) activity in the roots of *S. viminalis* of the control and phenanthrene treatment groups. Data presented are means (±SD), n=3 replications for control and treatment. A one-way ANOVA was applied, and $^{**}p<0.01$, $^{*}p<0.05$.

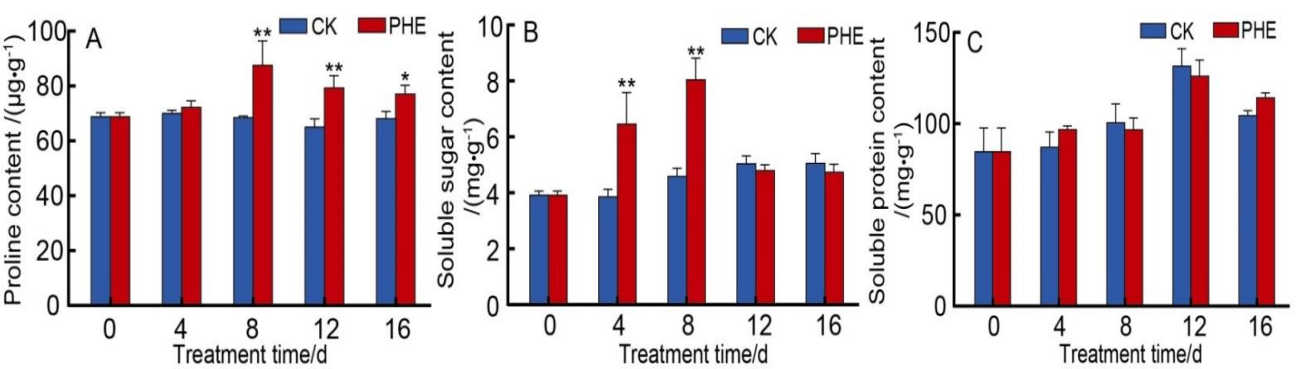

Fig. 3. Changes in proline (A), soluble sugar (B), and soluble protein (C) content in the roots of *S. viminalis* of the control and phenanthrene treatment groups. Data presented are means (±SD), n=3 replications for control and treatment. A single-way ANOVA was applied, and $^{**}p<0.01$, $^{*}p<0.05$.

Effect of PAH stress on gene expression in willow roots with GO analysis: According to GO analysis, phenanthrene had effects on *S. viminalis* in terms of cell components, molecular functions, and biological processes. A total of 45 functional groups including cellular component, molecular function and biological process were annotated. Ten functional groups, namely, cell membrane, membrane part, intrinsic components of membrane, catalytic activity, oxidoreductase, ion binding, cation binding, metabolic process of single organism, single tissue process, and stress response, were more distinct from the control in the number and significance of different genes (P-value≤0.05, Q-value≤0.05). There were 423 differentially expressed genes in the cell membrane, some of which were transporter genes. The CAT6 gene encoding the cationic amino acid transporter, aquaporin genes PIP1-2, PIP1.1, PIP2-7, PIP2-4, PIP2-2, and PIP2-1, and the iron transporter gene ZIP10 were downregulated under phenanthrene stress for 24 h. The potassium channel genes AKT1 and HAK5, phosphate transporters PHT1-4, PHT2-1 and Slc37a2, magnesium chelatase subunit HLH, magnesium transporter MRS2-11, and plasma membrane calcium transport ATPases ACA4 and ACA12 were also significantly decreased. Stress response includes enzymes related to antioxidant, sucrose synthesis and lignin synthesis. In terms of antioxidant stress, among 46 genes encoding peroxidase, 19 genes were upregulated and 27 genes were downregulated. The cytochrome P450 family showed significant changed in oxidoreductase metabolism, among which several key enzymes involved in phenylpropanoid synthesis were downregulated. For example, 17α-hydroxyprogesterone deacetylase CYP75A2 and CYP71BL3 and hydroxylase CYP84A1 were declined at 24 and 36 h. In ion binding, the cell wall-associated receptor kinases WAK1 and WAK2 declined at both time points.

Effect of PAH stress on gene expression in willow roots with KEGG analysis: In this study, KEGG enrichment analysis of different genes was performed according to the screening criteria of p-value≤0.05 and Q-value≤0.05. At 24 and 36 h, 35 and 24 significantly changed metabolic pathways were identified between the control and treatment groups (supplementary file). Among them, phenylpropanoid synthesis, nitrogen metabolism, and ABC transporters were downregulated and related to the toxic effect of phenanthrene on willow.

Changes of phenylpropanoid synthesis pathway under PAH stress: As shown in Fig. 4, lignin synthesis was significantly downregulated. The first step was the process of cinnamic acid formation from L-phenylalanine, which was catalyzed by phenylalanine lyase (Pichersky *et al*., 2006). Phenylalanine ammonia lyase genes PALG2B, PALG4 and PALG1 were downregulated at 24 and 36 h. In the later branching network, 4-coumarate CoA ligase 4CL1, caffeic acid 3-O-methyltransferase HOMT3, cinnamyl-CoA reductase CCR1, caffeyl-CoA CCoAOMT2, and caffeyl-shikyl-esterase CSE were downregulated at both time points. The polymerization of lignin monomers into macromolecule lignin requires the catalysis of peroxidase (POD) and laccase (LAC) (Chen *et al*., 2002). In the present work, cinnamol dehydrogenase CAD was upregulated at 24 and 36 h, and CAD1 and CAD6 were downregulated at 36 h. Among 46 genes encoding peroxidase, 19 genes were upregulated for oxidative stress to phenylalanine and 27 genes were downregulated that inhibit lignin formation. Laccase genes LAC6, LAC1, and LAC17 were downregulated at 24 h, which may be due to the damage of phenylalanine, while LAC5, LAC7, LAC14 and LAC22 were upregulated, which may be related to metabolism due to contamination. At 36 h, all genes except LAC22 were downregulated. Moreover, Glucosyltransferase GT5 was significantly downregulated.

Changes of nitrogen metabolism under PAH stress: In nitrogen metabolism (Fig. 5), cyanate lyase synthase gene CYN and glutamate dehydrogenase (NAD(P)+) gene GDH2 were upregulated at 24 h. The genes of the iron redox protein nitrite reductase NIA1, nitrate and nitrite transporter NRT2.1, glutamylamine synthetase GLN2, and glutamate synthetase GLT1 decreased at 24 h. At 36 h, glutamine synthetase GLN2, glutamate synthase GLT1, ferredoxin glutamate synthase Fd GOGAT and nitrate and nitrite transporter NRT2.1 were downregulated.

Changes of ABC transporters under PAH stress: Genes of ABC transporter families were altered. Among them, auxin transporters ABCG14 (Ko *et al*., 2014) and ABCB19 (Murphy, 2017) were significantly downregulated under phenanthrene stress. ABCA2, ABCA7, and ABCG11 were upregulated at the two time points. ABCB25 was upregulated 13.96 and 4.33 times in phenanthrene treatment group compared tocontrol group, at 24 h and 36 h.

Changes of osmoregulation under PAH stress: In this study, some genes related to osmoregulation were significantly changed (Table 1). Sucrose synthase SUS3, phosphosucrose synthase SPS4 and alpha-trehalase *TRE1* were significantly increased at 24 h and 36 h. Meanwhile, the sucrose transporters SWEET16 and SUC2 located on the cell membrane were significantly upregulated. Moreover, the key enzymes delta-1-pyrroline-5-carboxylate synthetase P5CS in the glutamate synthesis pathway of proline and rolyl 4-hydroxylase *P4H1* were remarkably upregulated at 24h and 36h.

Table 1. Key genes involved in osmotic regulation under phenanthrene stress.

KO number	Gene function	Gene symbol	log2(fc) (24h)	log2(fc) (36h)
K00695	Sucrose synthetase	*SUS3*	1.0157	0
K00696	Sucrose-phosphate synthase	*SPS4*	1.9706	1.7393
K01194	Alpha-trehalase	*TRE1*	2.0067	1.2230
——	Bidirectional sugar transporter	*SWEET16*	1.7703	1.9893
K12657	Delta-1-pyrroline-5-carboxylate synthetase	*P5CS*	1.0710	1.0732
K00472	Rolyl 4-hydroxylase	*P4H1*	1.7236	1.8762

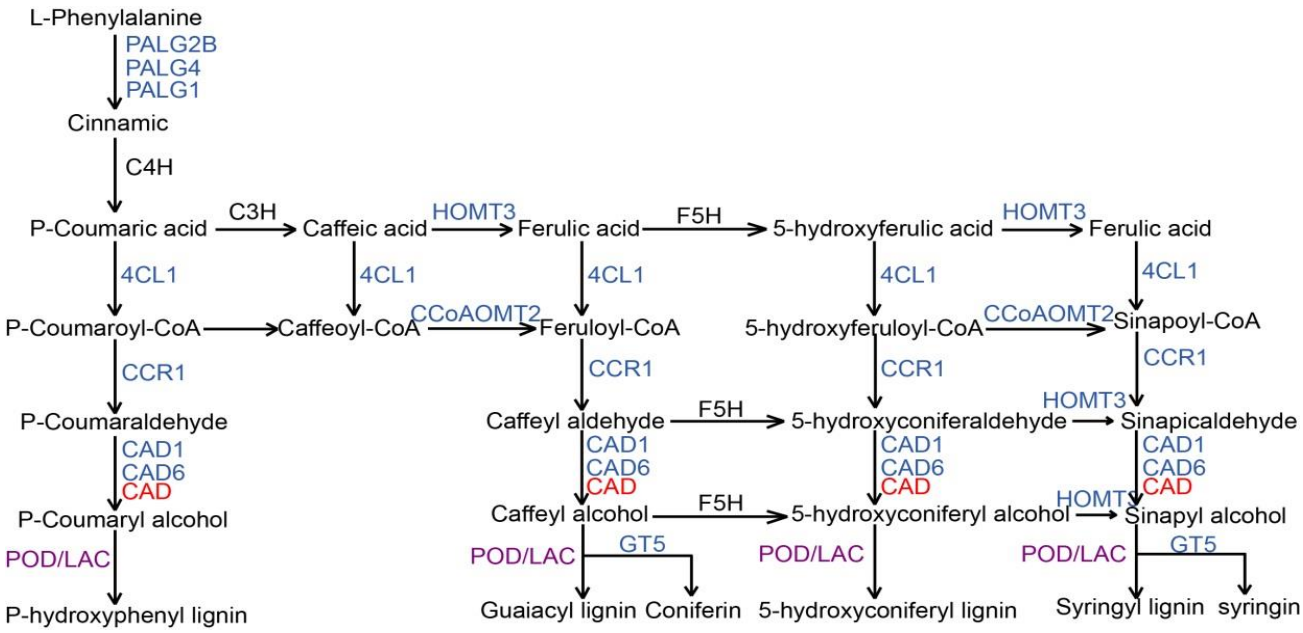

Fig. 4. Gene expression of phenylpropanoid biosynthesis in the roots of *S. viminalis* under phenanthrene stress. In the figure, gene names in blue represent significant downregulation (p<0.05), the red represents significant upregulation (p<0.05), and gene names in purple represent encoding peroxidase/laccase genes that were partially upregulated or partially downregulated. POD: Peroxidase, LAC: Laccase.

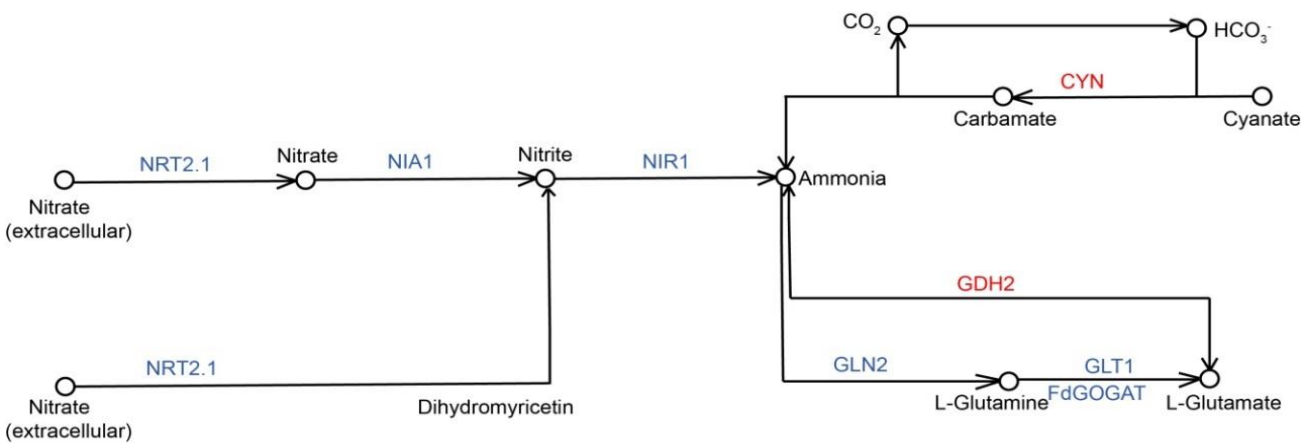

Fig. 5 Nitrogen metabolism under phenanthrene stress. In the figure, gene names blue represent significant downregulation (p<0.05), and the red represent significant upregulation (p<0.05).

Discussion

Antioxidant metabolism: Excessive reactive oxygen species (ROS) accumulation in plant cells can cause oxidative stress, leading to protein denaturation, lipid peroxidation, and nucleotide degradation, which can lead to cell damage and ultimately cell death. The induction of oxidative stress by PAHs in plants is a known effect (Pašková *et al*., 2006). In this work, the MDA content continued to increase after phenanthrene treatment of willow roots compared to the control. This indicated that the cell membrane of *S. viminalis* may be damaged due to the accumulation of ROS. $O_2^{\bullet-}$ was the main reactive oxygen, and it had a negative effect on plant roots.

Studies have shown that antioxidant enzymes have different responses to PAHs, which vary according to plant species and PAH concentrations. For instance, SOD and POD activity increased after Arabidopsis thaliana was treated with 0, 0.25, and 0.5 mM phenanthrene. With an increasing fluoranthene concentration, SOD activity in the leaves of Arabidopsis thaliana was elevated (Liu *et al*., 2008). Salehi-Lisar *et al*., (2015) confirmed that fluorene toxicity caused oxidative stress of *Triticum aestivum*, Medicago sativa, and Helianthus annus, as shown by MDA accumulation, and CAT could be an important enzyme involved in plants' resistance to tress. This may be because different species have dissimilar enzyme responses to cope with phenanthrene stress. In this study, SOD and POD activity in roots increased only at day 4, and CAT activity continued to increase for 16 days of phenanthrene treatment and played an important role in alleviating long-term oxidative stress. As a result of CAT being the main peroxide scavenging enzyme in plant cells, and the changes in CAT in roots were consistent with the decrease of H_2O_2 under phenanthrene stress for 8-16 d, we concluded that CAT was the positive enzyme involved in detoxification of H_2O_2 induced by phenanthrene toxicity in roots of *S. viminalis*.

Osmosis regulation: When plants are subjected to osmotic stress, the content of soluble sugars, including sucrose, will be increased to prevent a large amount of passive dehydration of cells to maintain their turgor pressure and maintain the stability of cell structure. Phosphosucrose synthase (SPS) and sucrose synthase (SS) are the main enzymes related to sucrose metabolism in higher plants. SPS is the main enzyme for sucrose synthesis, while SS mainly plays the role of sucrose decomposition in plant cells (Castleden *et al*., 2004). It has been reported that the soluble sugar content of *Kandelia candel* (L.) Druce and Arabidopsis leaves increased with an increase in PAH concentration (Lu, 2002; Yin, 2011). In this work, the soluble sugar content increased at 4 and 8 d, sucrose-phosphate synthase *SPS4* and bidirectional sugar transporter *SWEET16* were upregulated remarkably at 24 h and 36 h, indicating that sucrose synthesis and transport were enhanced under phenanthrene stress in the roots of *S. viminalis*.

Proline is not serving as a compatible osmotic pressure, but can also detoxify reactive oxygen species by forming stable complexes. Sivaram *et al*., (2019) concluded through metabolomics that pyrene induced a significant increase in proline content in maize leaves. In this study, proline content increased significantly at 8-16 d under phenanthrene stress, and the delta-1-pyrroline-5-carboxylate synthetase P5CS and Rolyl 4-hydroxylase *P4H1* were up-regulated at 24 h and 36 h, demonstrating a sign of osmosis stress in the roots of *S. viminalis* by PAH.

Furthermore, transcriptome sequencing results demonstrated that aquaporin genes PIP1-2, PIP1.1, PIP2-7, PIP2-4, PIP2-2, and PIP2-1 were significantly downregulated under phenanthrene stress. Aquaporin is regulated by pH, Ca^{2+} concentration, heteromerization, reactive oxygen species, and protein phosphorylation. In this work, reactive oxygen species were accumulated in the roots, causing some aquaporin genes to decrease, indicating that root cells rapidly change their water permeability to respond to phenanthrene stress and avoid toxicity while affecting water transport of plant cells.

Phenylpropanoid synthesis: Among the secondary metabolites, phenylpropanoid is involved in the antioxidant activity of the cell wall and the biosynthesis of lignin, which plays an important role in plant responses to abiotic stress (Bonawitz *et al*., 2011). Phenylpropanoid polymers, including lignin, cork and tannins, play a crucial role in improving the stability and robustness of plants under environmental stress (mechanical damage, pathogens, drought, etc.). As a mechanical barrier, lignin content in the cell wall can enhance the ability of anti-fungal penetration and anti-enzymatic digestion, protect host cells from degradation of fungal secretase, and prevent the diffusion of water and nutrients to pathogens. In this work, many key genes in the lignin synthesis pathway were significantly downregulated at both time points, implying that phenanthrene affected lignin synthesis, negatively affecting the barrier of lignin formation in roots, and making plant roots vulnerable to pathogens.

Mineral element transport and metabolism: Multiple studies have shown that PAHs have inhibitory effects on plant growth. When the pyrene concentration in soil is greater than 50 $mg \cdot kg^{-1}$, the fresh weight of roots, stems, and leaves of spinach at the seedling stage is significantly decreased, and the plant height is decreased. When the pyrene concentration in soil is greater than 100 $mg \cdot kg^{-1}$, the dry weight of spinach roots and stems and leaves is inhibited during harvest (Cai, 2009). In the present study, the iron ion transporter, nitrate/nitrite transporter, potassium ion channel, phosphate transporter, magnesium transporter and plasma membrane calcium transport ATPase genes were all significantly downregulated, indicating that the absorption of mineral elements may be inhibited, which leads to the slow growth and development of *S. viminalis*.

Nitrogen is an essential macroscopic element affecting plant growth and development. It connects the structure of numerous organic molecules, such as amino acids and proteins. In the absence of nitrogen, plant productivity decreases; thus, plant growth is limited (Luo *et al*., 2013a, 2015; Zhang *et al*., 2011). Plants usually obtain nitrogen from ammonium ions and nitrate (Hassan *et al*., 2008; Luo *et al*., 2013b). Nitrate ions absorbed by plants are catalyzed by nitrate reductase (NR) and nitrite reductase (NiR) to be degraded into ammonium ions and combined with organic molecular structures (Fontaine *et al*., 2006; Mishra & Dubey, 2011). Studies on nitrogen metabolism under PAH stress have not been reported, but conclusions can be made based on changes in genes related to nitrogen metabolism. Nitrate and nitrite transporter NT2.1 was downregulated at 24 and 36 h, restricting the transport of extracellular nitrate and nitrite, as the main factor decreasing nitrogen metabolism. Glutamine synthetase GLN2, glutamate synthase GLT1 and ferredoxin glutamate synthase Fd GOGAT were also downregulated at 36 h, indicating that the process of nitrate reduction to ammonia was blocked, which affected ammonia assimilation.

ABC transporters: ABC proteins comprise one of the largest ancient protein families and can use the energy released by ATP hydrolysis to transport various substrates, including lipids, hormones, mineral ions, peptides, secondary metabolites, and exogenous substances (Jungwirth & Kuchler, 2006). In the present research, auxin transporters ABCG14 and ABCB19 were downregulated, indicating that auxin transport may be inhibited. ABCB25 is associated with vacuolar sequestration of Cd or effusion from the symplast to the apoplasmic body (Yu *et al*., 2019). Since plants have a certain commonality in metabolism and transport of exogenous substances (organic and inorganic pollutants) (Weisman *et al*., 2010; Amrani *et al*., 2015) the large increase in ABCB25 may be related to the transport of phenanthrene or its metabolic intermediates. In addition, ABCA2 and ABCA7, which are closely related to plant stress and can alleviate the damage caused by hypoxia stress, salt stress, and heavy metal stress and are located in the cell membrane, were also elevated. According to GO function analysis, both were involved in the transport of polyamines. The up-regulation of ABCA2 and ABCA7 may be a defense response to phenanthrene stress.

Conclusion

The present study showed changes in physiology and molecular expression to further clarify the phytotoxicity of phenanthrene to the roots of *S. viminalis*. Phenanthrene induced the accumulation of $O_2^{\bullet-}$ so that MDA content increased, which indicated the peroxidation of root lipids. CAT, a positive enzyme involved in the detoxification of ROS, was induced by phenanthrene. Phenanthrene also caused osmosis stress in the roots, which was shown by the increase in proline and soluble sugar content, as well as related genes. Root cellulose synthesis was inhibited under phenanthrene stress because many key genes in the synthesis process significantly declined. The transport of some mineral elements might be hindered due to the downregulation of the iron transporter gene ZIP10, nitrate/nitrite transporter NRT2.1, potassium channel genes AKT1 and HAK5, magnesium transporter MRS2-11, and plasma membrane calcium transport ATPase ACA4 and ACA12. Furthermore, the downregulation of auxin transporters ABCG14 and ABCB19 was adverse to the transport of auxin.

Overall, PAHs are toxic to plant roots in several ways that inhibit plant production and harm the ecological environment. Therefore, understanding the phytotoxicity of PAHs to plants is the basis for improving plant resistance in contaminated soil, and it is of great significance for expanding the application of woody plants in the remediation of PAH-contaminated soil.

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