

Effects Research of Environmental Hormone BPA and PAEs on the Expression of HSP70-9 Gene in *Spinacia oleracea*

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Abstract

Objective: In order to explore the impacts of environmental hormones (EHs) on plants, the relationship between EHs pollutants and *Spinacia oleracea* HSP70-9 expression was studied in this paper. The researchers studying HSP70 have primarily investigated high temperature stress, low temperature stress, water stress and other environmental factors, such as heavy metals, wind stress, salt stress. However, as a type of environmental pollutant, EHs may affect the growth and development of plants.

Methods: Two typical EHs, PAEs (phthalates esters) and BPA (bisphenol A), were selected for the study. RT-qPCR was used to study HSP70-9 expression at the transcriptional level under single and combined pollution conditions in spinach seedlings.

Results: The result of the present study showed that, the spinach HSP70-9 expression is downregulated with single and combined treatments of EHs.

Conclusion: The conclusion is that BPA and PAEs had certain effects on the expression of HSP70-9 in spinach.

Keywords: HSP70; *Spinacia oleracea*; Gene expression; BPA; PAEs

Introduction

Environmental hormones (EHs) are compounds present in the environment which can interfere with normal metabolic activities in organisms [1-5]. EHs are divided into two major heads namely, degradation-resistant organic halogens, which includes pesticides, industrial compounds, heavy metals, organic solvents, estrogens, and second category includes plant growth regulating agents [6,7]. EHs can cause disorders in organisms (animals), such as cancer, genetic mutations, obesity, infertility, Alzheimer's disease and Schizophrenia [2,4-8]. However, little is known regarding the harm EHs pose toward plants.

Current researchers who study EHs impact on organisms continue to increase, and have mainly focused on the influence of heavy metals on aquatic animals and plants [9-12], pesticides on insects [11], and EHs on organism genotoxicity [5]. The plasticizers, BPA for clear plastics and PAEs in hard plastics, have some estrogenic activity and anti-androgenic activity [4]. With the improvement of living standards, plasticizers such as BPA and PAEs are appearing more frequently in our daily lives, and their impacts to organisms are becoming increasingly prominent [4]. BPA and PAEs mimic the functions of estrogens and anti-androgens [4]. Nonyl phenol (NP), BPA, EE (17 α - ethinyl estradiol) and DEHP (one type of PAEs) treatments caused a significant increase of HSP70 in chironomids (*Chironomus tentans*) [11]. With the exception to heavy metals researchers, there are few current researchers studying EHs in plants, and whether BPA and PAEs impact spinach remains unknown.

Plants experiencing adversity can produce some novelty proteins or shut down some proteins biosynthesis to resist the impact of a single stress or combined stress [13-15]. Although heat shock proteins (HSPs) were discovered and named after their response to thermal stress [16], HSPs are not only regulated by temperature stress, but induced by a range of other environmental stress, such as salinity, cold and heavy metals [17]. Generally it is agreed with that HSPs (especially the HSP70 and HSP90 families) play an important role in alleviating the damage caused by various environmental stresses [13,18]. HSPs can be divided into five major families: HSP100, HSP90, HSP70, HSP60 and

small HSP family [19,20]. Among these families, HSP70 family is the highly conserved and the most researched gene family [11,15,17,20-24]. Studies have demonstrated that heat stress [15,18-20,24-32], Cold stress [15,24,26,29,30], water stress [22,25,26,33,34], oxidative stresses [19,20,22], heavy metals [9,12,13,35], salt stress [13,19,20,22,34], light stress [27], and chemical contaminants [11,15,18,19,25,35-37], among others factors, can induce HSP70 [11,14,22,24,35-41]. In plants, HSP70 are categorized into four major subgroups based on the unique and highly conserved C-terminus motif, including the cytosolic, endoplasmic reticulum (ER), mitochondrion (MT) and chloroplast (CP) group [25,42]. However, studies support that HSP70 located specific subcellular has different responses to different environmental stresses [14]. The product of HSP70-9 located in chloroplasts in spinach [31]. Chloroplasts are the important organelles in plants and conduct the organic energy transformation from solar energy. Otherwise, many studies have shown that the activity and quantity of plant chloroplasts decreased under many stresses. So we want to study whether the HSP70-9 mRNA expression changes under the conditions of BPA and PAEs pollution.

Spinacia oleracea has broad leaves and rapid growth and is rich in metal elements and nutritional characteristics; therefore, this plant is an important vegetable in daily life [9,43]. We have selected BPA and PAEs to study in both single and combined contaminant conditions on spinach, measuring the HSP70-9 expression. The result may provide a reference

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value for environment pollution control and ecological restoration and may be especially pertinent to the impact of environmental pollutants on crops.

Materials and Methods

Preparation of EHs solutions of BPA and PAEs

The PAEs solution was prepared from DBP (dibutyl phthalate) and DEHP (diethylhexyl phthalate) by volume ratio of 9:1. Gradient dilution of the PAEs solution was used to obtain five concentrations ($\text{mg}\cdot\text{L}^{-1}$): 0, 0.5, 5, 10, 50 (as specified in "Research of Chinese Environment PAEs Compounds Contamination and Soil Environmental Quality Standard"). The BPA solution was prepared using gradient dilution to obtain five concentrations ($\text{mg}\cdot\text{L}^{-1}$): 0, 0.05, 0.5, 5, 50 (as specified in "Summarize of Environmental Behavior for Typical Environmental Hormones BPA"). We carried out a pairwise comparison of the combined BPA and PAEs solution with the BPA and PAEs single solutions. There were 25 group-solutions of BPA and PAEs single solutions and combined solutions, as shown in Table 1. All of the solutions were sterilized for 20 min at 120°C and used for treatment after cooling.

Plant materials

Spinach (*Spinacia oleracea* L.) seeds were purchased from the Shenyang Agricultural University seed company in China. The seeds were disinfected in 0.1% KMnO_4 for 15 min and rinsed with distilled water. Dried seeds were cultured in an intelligent light incubator and fertilized with 25 different concentrations of EH solutions. The culture temperature was $15^\circ\text{C} \pm 1^\circ\text{C}$ and 12 h light after 12 h dark. Approximately 4 weeks later, experiments were initiated when the seedlings developed to their true leaf stage.

RNA extraction and RT-PCR

Total RNA was extracted from different Spinach leaf samples with Trizol Reagent (BioTeke Biotechnologies Inc., Beijing, China). We prepared the RNA according to the method recommended by manufacturers (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The cDNA strand was synthesized resulting in a final volume of 20 μL , including 1 μL total RNA, $5 \times 2 \mu\text{L}$ gDNA Buffer, $10 \times 2 \mu\text{L}$ Fast RT Buffer, 1 μL RT Enzyme Mix, 2 μL FQ-RT Primer Mix, and 12 μL DEPC-treated water. The RT-PCR products were used for qPCR or stored at -20°C .

qPCR

All qPCR primers were designed using Primer Premier 5 and Oligo 7 software (Table 2), and synthesized by Shanghai Sangon Corporation (all primers had their purity qualified by mass spectrometry).

qPCR was used to detect the gene expression of Spinach HSP70-9. 16s rRNA was used as the reference. The PCR mix had a total volume

of 20 μL , containing 0.5 μL cDNA, 0.4 μL of 10 μM forward primer, 0.4 μL of 10 μM reverse primer, 10 μL of 2x Power SYBR Real-timePCR Premixture (BioTeke Biotechnologies Inc., Beijing, China), and 8.7 μL sterile water. The reaction program was as follows: initial denaturation at 95°C for 2 min, 45 cycles at 95°C for 20 sec, 56°C for 20 sec, 72°C for 20 sec. The melting curve procedure was as follows: denaturation at 95°C for 15 sec, and an increase of 65°C up to 95°C at a rate of $0.5^\circ\text{C}/\text{s}$. PCR amplification was performed in the ABI StepOne™ Real-Time PCR System (ThermoFisher, USA).

The $2^{-\Delta\Delta\text{CT}}$ method was used to calculate the resulting data [44]. The experimental results were performed by SPSS 18.0 (SPSS Inc., USA) software for One-Way ANOVA and the LSD (Least Significant Difference) test was used to compare the differences among means of treatments. Differences were considered statistically significant at $P < 0.05$ and very significant at $P < 0.01$.

Results

Spinach HSP70-9 expression influenced by single pollutants of BPA and PAEs

To test whether spinach HSP70-9 gene expression was regulated by BPA and/or PAEs, total RNA was isolated from spinach seedling, which had been treated (except the control) with BPA and/or PAEs and subjected to RT-qPCR. Figure 1 shows the relative levels of *Spinacia oleracea* HSP70-9 expression when influenced by BPA (A) and PAE (B) single pollution. These results indicate that HSP70-9 exhibits similar expression patterns under the single BPA and PAEs contaminant treatments, and HSP70-9 is significantly downregulated when exposed to both. Moreover, when the same HSP70-9 exposed to the single BPA pollution treatment (Figure 1A) and single PAEs pollution treatment (Figure 1B), with the exception of the B_5 treatment group, HSP70-9 has been shown to significantly downregulated in the treatment groups compared with the control groups. In addition, with the exception of B_{50} treatment group, the expression level of HSP70-9 increased with the increase of EHs concentration in each treatment group. Finally, HSP70-9 has minimal expression under the B_{50} and $P_{0.5}$ concentrations.

Spinach HSP70-9 expression influenced by combined pollutants of BPA and PAEs

In general, environmental pollutants usually coexist together with other environmental pollutants. So it is interesting to study the combined effects of BPA and PAEs on spinach seedling. The relative expressions of spinach HSP70-9 appear to be influenced by the treatment of combined BPA and PAE contaminant (Figure 2A-2D). All groups showed the same downregulated expression patterns. In addition, the expression level of HSP70-9 increased with the increase of EHs concentration in each treatment group. Finally, HSP70-9 has minimal expression under the $P_{0.5}B_{0.05}$, $P_5B_{0.05}$, $P_{10}B_{0.05}$ and $P_{50}B_{0.5}$ combined pollutions.

Discussion

As a category of environmental pollutants, EHs are bound to affect

EHs Solutions/ $\text{mg}\cdot\text{L}^{-1}$	BPA				
	P_0B_0	$B_{0.05}$	$B_{0.5}$	B_5	B_{50}
PAEs	$P_{0.5}$	$P_{0.5}B_{0.05}$	$P_{0.5}B_{0.5}$	$P_{0.5}B_5$	$P_{0.5}B_{50}$
	P_5	$P_5B_{0.05}$	$P_5B_{0.5}$	P_5B_5	P_5B_{50}
	P_{10}	$P_{10}B_{0.05}$	$P_{10}B_{0.5}$	$P_{10}B_5$	$P_{10}B_{50}$
	P_{50}	$P_{50}B_{0.05}$	$P_{50}B_{0.5}$	$P_{50}B_5$	$P_{50}B_{50}$

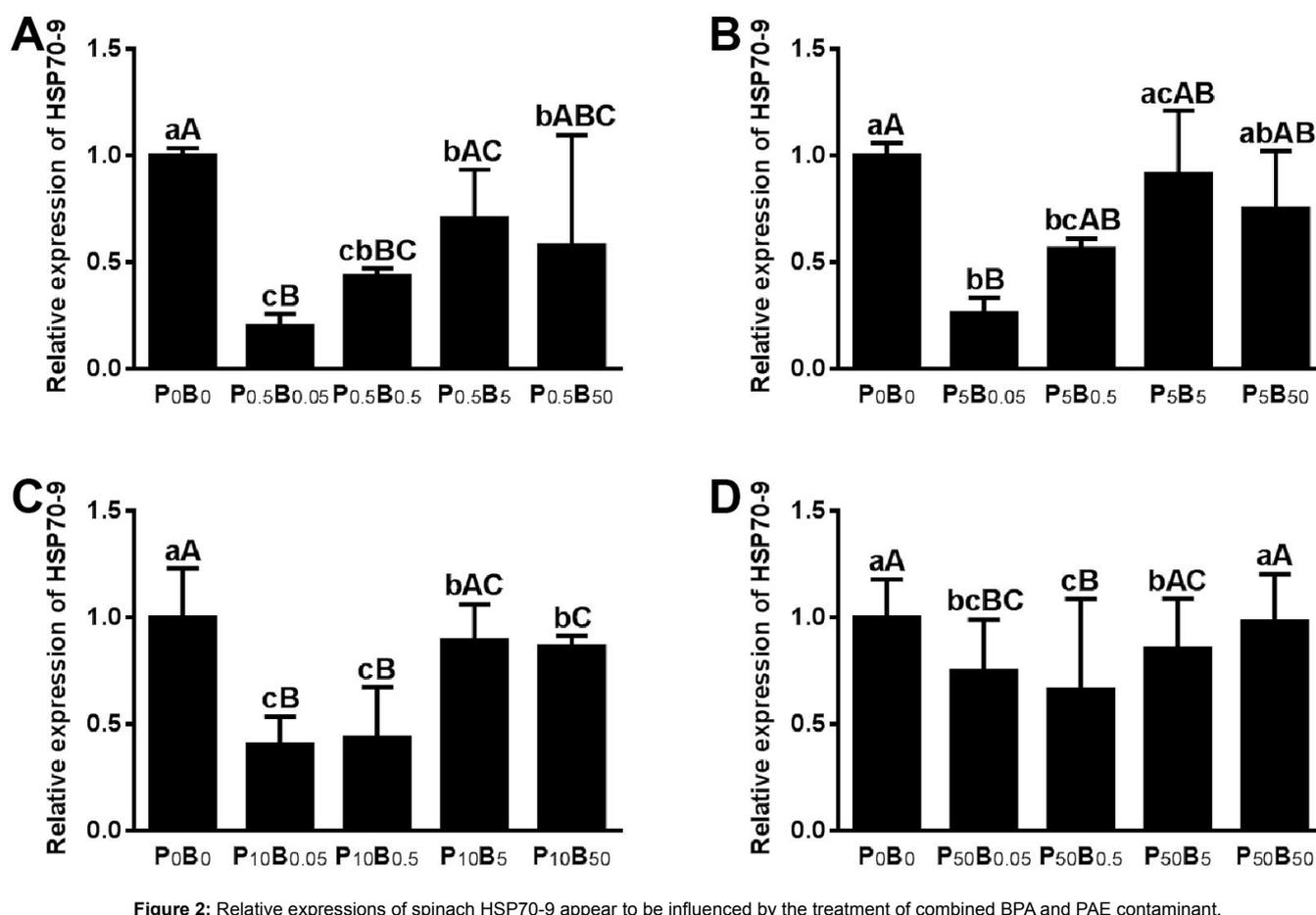
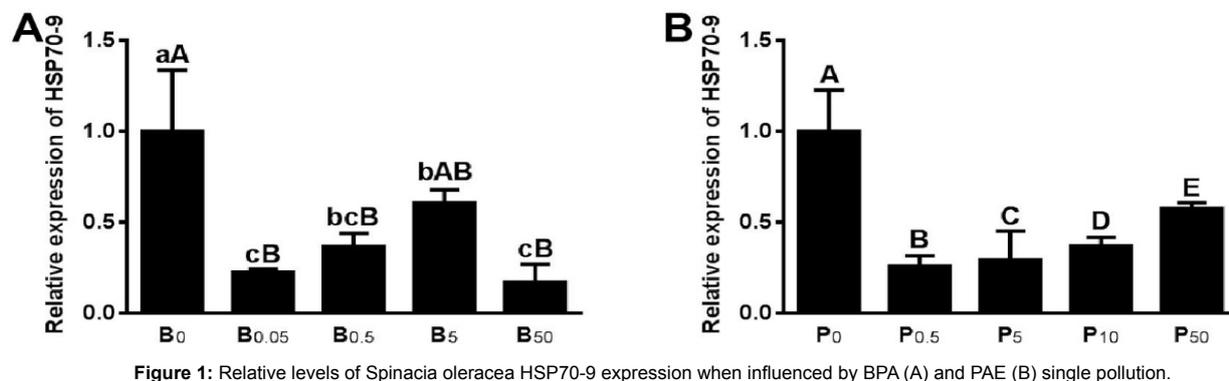
The letters B and P mean BPA and PAEs respectively and subscripts indicate concentrations. Such as, $P_{0.5}$ is as a representative of PAEs single contamination of $0.5 \text{ mg}\cdot\text{L}^{-1}$; $P_{0.5}B_{0.05}$ is as a representatives of PAEs and BPA combined pollution with concentrations of $0.5 \text{ mg}\cdot\text{L}^{-1}$ and $0.05 \text{ mg}\cdot\text{L}^{-1}$. The same to that are below.

Table 1: Preparation of different concentrations solution for BPA and PAEs.

Gene	Sequence (5'-3')	Length, bp	Product, bp	GenBank ID
HSP70-9	F: CTTCTATTGGATGTTGCACCT	20	187	AF035456
	R: TAAGATCGAATTTCCCGAGA	21		
16S rRNA	F: CCCAACGTCAGTTTTTCTATTTTGA	25	72	J01440(Chen and Arora, 2014)
	R: CCACGAGCCTCTTATTCATTCTC	23		

F: Forward; R: Reverse.

Table 2: Primer sequences for the qPCR amplification of specific genes.



the expression (up or down regulation) of certain genes in organisms living in harmful environments, whether directly or indirectly [11]. It may therefore be reasonable to assume that there are genetic mechanisms that resist or adapt to this type of adverse environment. However, each organism experiences distinct pathways for genetic inheritance and evolution, so EHs-induced expression varies among genes, gene number and the forms of impact.

Traditionally, the impact of environmental stressors on plants is studied mainly by measuring plant height, root length, biomass

and various methods for determining physiological and biochemical parameters. However, many recent studies on plant stress have been carried out using molecular biology techniques [29,45,46]. In this paper, a more sensitive molecular biology method, RT-qPCR, was used in the present study to detect spinach HSP70-9 expression at a transcriptional level in response to single and combined treatments of EHs.

Lee et al. suggested that, the response of the HSP70 expression by BPA and DEHP (one of PAEs) exposure was sensitive to low concentrations in *Chironomus tentans* [11]. Duan et al. found that

salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) treatments had no influence on chloroplastic HSP70 expression in wheat [18]. Spinach HSP70 have sensitive response to heat [26-28,32], cold [28,47], water [26], wound [26] and heavy metal stress [9,12]. As showed in Figure 1 and Figure 2, the present study quantitatively analyzed the expression of spinach HSP70-9 at the transcriptional level in response to the EHs, BPA and PAEs in both single and combined pollution treatments. The HSP70-9 expression in all treatment groups were all downregulated compared with the control groups.

Plants treated by EHs or other stress have a decrease of chlorophyll content, biomass [9,12,22,48,49]. It is suggested that the plant life activities have been seriously affected in the adverse environment. Researchers suppose that chloroplast has damaged since the chlorophyll content decreased and newly synthesized chloroplastic HSP70 may fail to enter chloroplasts after stress and may be degraded in the cytoplasm [31]. Thus, the downregulated expression of spinach HSP70-9 which located in chloroplast seems to be reasonable.

Although HSP70 members share a conserved structure and a similar mechanism, they have different mechanisms in forming chaperone complexes and performing their function in stress [30,50]. The specific roles of individual HSP70 proteins are likely to be determined by their location in different subcellular compartments [17,30], the cytosolic HSP70 prevents protein aggregation, assists *de novo* protein folding and maintains the organellar precursor proteins in an import-competent stage, and the ER, mitochondrial and chloroplastic HSP70 proteins are involved in precursor protein import and translocation [23,31,51,52]. So we assume that organelles and cytosolic HSP70 of *Spinacia oleracea* possibly work through different molecular mechanisms in responding to specific stress.

As a chaperone molecule, HSP70 performs various functions, such as folding unfolded proteins, targeting and degrading denatured proteins, monitoring proteins for correct folding, and transporting and positioning the precursors of mature proteins [17,53]. Masand et al. found that, proteolytic activity was significantly increased in wild type compared to transgenic plants under different abiotic stresses in *Arabidopsis thaliana* [22]. The total protein is decreased under the treatment by BPA and PAEs single and combined solutions in Glycine max (data not show). Heavy metal concentrations also had a significant adverse impact on total protein in *Spinacia oleracea* [9]. As shown in Figure 1, the spinach HSP70-9 expression shows an upward trend as BPA and PAEs concentrations increase. This finding suggests that the increased EHs lead to the denaturation of spinach seedling proteins. Since chaperone proteins may play a vital role in the cell homeostasis, the increased expression of HSP70-9 could indicate chaperone proteins' role in restoring denatured proteins and preventing the degeneration of aggregated proteins [46], among other functions, after exposure to EHs.

Research shows that the impacts caused by combined pollutants were more serious than a single pollution [1]. Furthermore, the coexistence of various EHs is a current environmental issue. Therefore, it is necessary to study the environmental impacts of combined EH pollutants on plants. Figure 2 shows the spinach HSP70-9 expression on transcriptional level under BPA and PAEs combined contaminants. It shows similar expression patterns to the expression patterns displayed under single EH pollutions. The study of heavy metals pollutants treatments on spinach, including Cd, Pb and Zn, suggest that the impact of combined pollution is stronger than that of single pollutions, but weaker than the mathematical addition of two single pollutions' impact [9]. A study of Cd and NaCl pollutants suggest that the expression of

spinach HSP70 is significantly increased under the combined pollution, but unchanged under either individual pollution [13]. Comparing Figure 1 and Figure 2, HSP70-9 expression under combined pollutants appears lower than expression in response to single pollutions, with the exception of the P₁₀ BPA. This finding suggests that spinach uses different mechanisms to respond to combined and single EHs pollution [13], but the same response protein in both mechanisms is HSP70. The induction of HSP70 under environmental stress is regulated by heat shock transcription factors (HSF) and corresponding heat shock elements (HSE) in the promoters [24]. Therefore, it is necessary to study the mechanism of HSP70 in response to environmental stress from the molecular level by further study the interaction between HSF, HSE and HSP70.

Spinach response and resistance to these environmental stressors not only depend on specific proteins or signaling pathways, but the combined effects of a variety of proteins and signaling pathways [13]. Meanwhile, with the large variety of EHs and the wide functional range of pollutions, it is presumable that, the long-term effects of EHs on organisms will be a long and difficult task. Therefore, genomics, transcriptomics, proteomics and other similar methods should be used to explore the single and combined pollution response mechanisms of plants.

Conclusions

In this study, two common EHs, BPA and PAEs, were used to detect the expression of spinach HSP70-9 using RT-qPCR under different concentrations of single and combined pollution conditions. The results show that under BPA and PAEs, single contaminant conditions, spinach HSP70-9 expression shows an overall trend toward downregulation. Under the combined BPA and PAEs treatment conditions, all treated groups also presented downregulated HSP70-9 expressions; however, spinach HSP70-9 expression was higher in combined pollutant conditions than in single pollution conditions. As a type of responsive protein, spinach HSP70 has an important role in the resistance and adaptation mechanisms responding to adverse environments containing BPA and PAEs.

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Author Contributions

Conceived and designed the experiment: Peiyong Liu. Performed the experiments and analyzed the data: Wenxue Jia. Contributed reagents/materials/analysis tools: Wenxue Jia and Peiyong Liu. All authors were involved in writing the paper and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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