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Effect of cyclic aromatic hydrocarbons on the response of some stress genes and the activity of several antioxidant enzymes date palm leaves

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Abstract:
This study used 18-month-old date palm offshoots treated with a combination of polycyclic aromatic hydrocarbons (PAHs), including naphthalene, anthracene, and Benzo(a)Pyrene, at concentrations of 0, 10, 30, and 60 mg/kg. After 85 days of treatment, samples were taken from the palm leaves for biochemical tests and gene expression analysis. The results showed that treatment with PAHs increased the concentration of hydrocarbons, particularly aromatic hydrocarbons. Additionally, the treatment led to an increase in the concentration of carbohydrates and proline. For example, at a concentration of 60 mg/kg of PAHs, the carbohydrate content reached 14.71 ±0.57 mg/100 g compared to the control sample (9.88 ±1.05 mg/100 g), and the proline content was 8.32±0.99 µmol/g compared to 2.64±0.57 µmol/g in the control sample. However, treatment at this concentration decreased chlorophyll pigment and carotenoids compared to the control sample. Chlorophylls a and b reached 2.47±0.58 and 2.13±1.02 mg/g respectively, while the control sample reached 4.52±0.56 and 2.21±0.66 mg/g. Similarly, carotenoids decreased to 1.47±0.53 mg/g compared to 1.78 ±0.01mg/g in the control sample. The activity of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) also increased with increasing treatment concentration. For example, the SOD enzyme activity reached 5.42±0.59 units. min^{-1} at a 60 mg/kg concentration of PAHs compared to 2.23±1.005 units. min^{-1} in the control sample. The CAT enzyme activity reached 6.53±1.54 units. min^{-1}, and the control sample reached 4.32±0.56 units. min^{-1}. The POX enzyme activity was 48±1.00 units. min^{-1} compared to 29±1.02 unit. min^{-1} in the control sample. The malondialdehyde (MDA) concentration increased at 60 mg/kg of PAHs, accompanied by a decrease in the membrane stability index (MSI). However, as the treatment concentration decreased, the concentration of MDA decreased and the MSI increased. The gene expression analysis of date leaves treated with different concentrations of PAHs showed that the SOD gene had the highest expression level at the concentration of 60 mg/kg PAHs and reached 0.99- fold compared to the control treatment, which recorded 0.23- fold for this gene. The expression of other studied genes also increased in the concentration of 60 mg/kg PAHs compared to other treatment concentrations.
Keywords: antioxidant enzymes, heavy metals, gene expression, Phoenix dactylifera L.

Introduction:

Environmental degradation resulting from human activities leads to an imbalance in environmental systems, particularly in soil and agriculture. One of the most dangerous environmental pollutants that causes significant harm to the environment is polycyclic aromatic hydrocarbons (PAHs), due to their numerous and diverse sources. These sources include natural occurrences such as forest fires and volcanic eruptions, as well as anthropogenic sources like the mining industry, oil refining, coal tar, and the use of diesel and gasoline in vehicles (Lee et al., 2021).

Naphthalene and anthracene are aromatic hydrocarbon compounds with low molecular weight. Although they are considered toxic substances, they are not carcinogenic. The main source of these compounds is the incomplete combustion of fuel derived from petroleum and various industries, including dye, leather, and textile factories. Naphthalene and anthracene can be found in all environmental systems, indicating their accumulation. Naphthalene is often used as an indicator of pollution due to its different properties, such as solubility in water, abundance, and ability to be biodegraded by microorganisms. These pollutants enter plants through two pathways: either through adsorption by the roots and accumulation in the rhizosphere or through the leaves from the polluted atmosphere (Phale et al., 2019). When present in small quantities, these compounds stimulate plant growth (Jalal et al., 2021), but in abundance, they become stressful for the plant (Kaur et al., 2017).

Benzo(a)Pyrene (B(a)P) is a polycyclic hydrocarbon compound with a high molecular weight. It is considered highly dangerous and toxic due to its carcinogenic and genetically mutagenic properties, which harm animals and plant cells as well as crops. It is known to be lipophilic and is primarily generated through incomplete fuel combustion or thermal decomposition in low-oxygen environments (Voloshina et al., 2023).

The primary strategy for mitigating the effects of PAH pollution involves the accumulation of these compounds within plant tissues, while also activating anti-stress responses. When plants are exposed to stress, they endeavor to protect their cells from oxidative stress by maintaining optimal levels of reactive oxygen species (ROS) through an enzymatic defense mechanism, which includes SOD, CAT, POX, and other enzymes as well as a non-

enzymatic defense mechanism comprising vitamins E and C, carotenoids, flavonoids, and others (Pan et al., 2019).

Threonine specific kinase/serine (Salt Tolerance Kinases) (*STKs*) are a group of protein kinases that phosphorylate the OH group of serine and threonine. *STKs* are considered a large protein family and are essential in transmitting signals to stressed plants through phosphorylation The *STK* group consists of different protein kinases, including mitogen-activated protein kinase (MAPK), calmodulin-dependent protein kinase (CAM), protein kinases A (PKA), C (PKC), and phosphorylase kinase (PhK) (Janczarek et al., 2018). The pathogenesis-related protein 1 (*PR-1*) family belongs to the PR proteins, which are antimicrobial peptides (AMPs) classified into 17 families based on similarities in protein sequences, enzymatic activities, and biological features (Han et al., 2023). Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), also known as *PIP_2* or *$PI(4,5)P_2$*, is a minor phospholipid component of cell membranes. It is enriched at the plasma membrane, serving as a substrate for several important signaling proteins. *PIP2* also forms lipid clusters that sort proteins(Berny et al., 2016). The family of superoxide dismutase (*SOD*) genes plays an essential role in protecting plants from biotic and abiotic stress conditions, as well as contributing to plant growth and development. Activation of this enzyme is the first line of defense against the effects of ROS (Su et al., 2021).

Date palm (*Phoenix dactylifera* L.) is a perennial woody plant belonging to the Arecaceae family. Palm trees are commonly found in arid desert environments and are known for their ability to tolerate salinity and drought. As a result, this species often possesses genes that make them resistant to both biotic and abiotic environmental stresses. In addition to salinity and drought, palm trees also face significant challenges from oil pollution and heavy metal contamination. While they are capable of coping with these stresses, resistance to them often leads to a loss of certain desirable qualities, such as reduced productivity and a lack of fruit production (Meddich et al., 2018). To gain a deeper understanding of the impact of PAHs on date palm plants, it is important to study the ROS generated and the nature of stress genes as well as the extent of their influence on other biochemical traits. This knowledge will help in finding ways to mitigate the effects of stress and could potentially be used as a biomarker.

Materials and methods:

The experiments involved using 18-month-old palm offshoots treated with a combination of aromatic hydrocarbons, including naphthalene, anthracene, and B(a)P at concentrations of 0, 10, 30, and 60 mg/kg. Each treatment was replicated three times, and the experiments lasted 85 days. After the treatment, palm leaves were collected, washed, and sterilized with 70% ethyl alcohol. They were then air-dried, ground, and passed through a 2 mm sieve. Samples were taken to the laboratory for analysis of aromatic hydrocarbons using GC- MC. The extraction and quantification of PAHs were carried out using a modified version of the method developed by Grimalt and Olivé (1993).

Once the benzene extract containing PAHs was collected, it was evaporated aerobically in the laboratory. The resulting precipitate fraction was then dissolved. For the GC analysis, 10 µl of samples were injected into the GC machine along with 1 ml of n-Hexane. The concentration of the standard sample was set at 0.5 ppm, and the standard PAHs used were obtained from the American company ULTRA SCIENTIFIC. The injection was done using a Japanese splitless injection unit. The GC-MC device used in the analysis was manufactured by Agilent Company and was equipped with a flame ionization detector (FID). The device specifications and conditions were as follows: GC column type: DANY 05 (30 m×0.32 mm), mobile phase: N_2, flow rate: 1.5 ml/min, detection: FID @ 300C, temperature program: 110 °C @ 1/min)-190 °C @12/min-120 °C@6/min-250 °C @3/min.

Quantitative real-time polymerase chain reaction assay (reverse transcription):

Quantitative Reverse Transcription Real-Time PCR (RT-qPCR) assay was performed to measure mRNA levels of the genes indicated in Table (1). This assay provides a quantitative analysis of gene expression. The *ACTIN* gene was used as a standard regulatory gene to calculate gene expression levels.

Table (1) Target stress genes in date palm

Gene name	Gene Description	Primer sequence (5*3) Forward	Primer sequence (5*3) Reverse	

1-STKs	Serine threonine protein kinases STY8	TATGGCGGCTTATCTTTTGG	CTTGTTCGGAAGAGGAGGTG	
2-PIP2	Aquaporin PIP2-4	TCCCACGTCCCGGTTTT	GGACCATGAACACCGCAAA	
***3-ACTIN* (ref)**	Actin	TCAATGTGCCTGCCATGTATGT	GCGGCCGCTAGCATAGAG	
SOD	dismutase superoxide	TGGTTTGGGATTACTCGCCC	GCTCTTTGCCAGCCAGAGTA	
4-PR1	Pathogenesis related protein 1	GCAGACTCATACACTCTGGTGG	ACTCCATTGCACGTGTTCGCAG	

Total nucleic acids were extracted from plant tissues treated with hydrocarbon pollutants at different concentrations using the Total RNA Isolation kit provided by Promega. The extraction process involved following the steps provided in the kit.

Manufacturing of cDNA:

We used the Reverse Transcription System Goscrip kit prepared by Promega to manufacture cDNA that is complementary to the cDNA from the RNA samples of the study samples.

Quantitative Real-Time PCR (qRT-PCR) test:

A qRT-PCR test was conducted on the cDNA samples using the GreenStar qPCR dye, which reacts with the target genes in the Real-Time PCR device. The qRT-PCR reaction mixture was prepared for both the target genes and the standard type gene of the organization.

Assay of biochemical characteristics:

The activity of the POX enzyme was determined using the method mentioned in Zouari et al. (2016) and Kim and Yoo (1996), while the CAT enzyme was determined following the method described by Goth (1991). Total soluble carbohydrates and proline were determined using the method of Watanabe et al. (2000), with a standard glucose curve. The proline content in the leaf tissue samples was estimated based on the method of Bates et al. (1973). The MDA content and MSI were estimated using the method described by Heath and Packer (1968).

Results:

Effect of PAHs on gene expression level

Figure 1 and Table 2 show the effect of adding aromatic hydrocarbons on the level of gene expression for each of the genes *STK*, *SOD*, *PIP2*, and *PR1* shown in Table 1. There are significant differences in the study coefficients for the target gene *STK*, with the treatment of 60 mg/kg of PAHs recording the highest gene expression for the *SOD* gene at 0.99-fold, compared to the control treatment where the gene expression value was recorded at 0.23-fold. Additionally, the treatment with a concentration of 30 mg/kg recorded gene expression of 0.96-fold while the treatment with 10 mg/kg recorded 0.46-fold. For the *PR1* gene, the treatment with 60 mg/kg of aromatic hydrocarbons recorded the highest gene expression at 0.96-fold compared to the control treatment.

The *STK* and *PIP2* genes recorded the highest gene expression in the 60 mg/kg treatment at 0.96-fold. The treatment with a concentration of 30 mg/kg also recorded a significant difference in gene expression, with the expression of *PIP2* and *STK* genes reaching 0.61-fold and 0.96-fold respectively, compared to the control sample which had gene expressions of 0.04-fold and 0.23-fold, respectively.

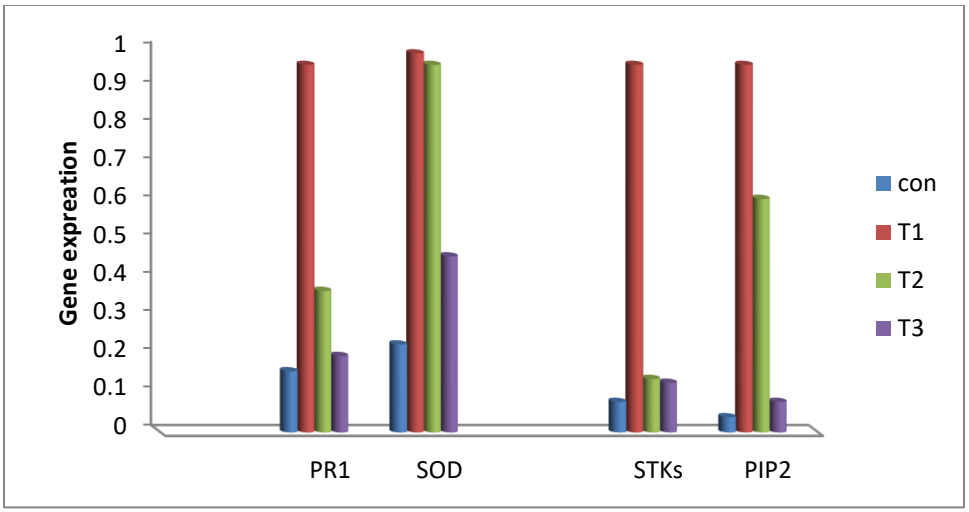

Fig. 1: The effect of different concentrations of PAHs on the expression level of *STK, SOD, PIP2*, and *PR1* genes. Con: Control Sample, T1: 60 mg/kg, T2: 30 mg/kg, T3: 10 mg/kg.

Table 2. The effect of different concentrations of PAHs on the expression level of *STK, SOD, PIP2*, and *PR1* genes.

Genes	*PR1*	*SOD*	*PIP2*	*STKs*
Control sample	0.16±0. 09^{c}	0.23±0.05^{c}	0.01±0.004^{c}	0.08 ±0.002^{c}
concentration of 60 mg/kg (PAHs)	0.96±0.04^{a}	0.99 ± 0.04^{a}	0.96 ±0.03^{a}	0.96±0.06^{a}
concentration of 30 mg/kg (PAHs)	0.37±0. 04^{b}	0.96 ±0.02^{a}	0.08±0.006^{b}	0.14±0.03^{b}
concentration of 10 mg/kg (PAHs)	0.20 ±0.02^{c}	0.46±0.07^{b}	0.04±0.005^{c}	0.13±0.01^{b}

Different letters indicate a significant difference of $P \leq 0.05$ of the samples compared to the control.

Effect of PAHs on the biochemical indices of palm leaves

Carbohydrates and proline content

The results of Table 3 show that treatment with PAHs at a concentration of 60 mg/kg was stressful for the plant and led to a significant increase in the content of carbohydrates and proline compared to the control sample. The carbohydrate content and proline content reached 14.38±0.57 mg/kg and 8.32±0.97 µmol/g, respectively. The control sample had a concentration of carbohydrates and proline of 9.88 ±1.05 mg/kg and 2.64 ±0.57 µmol/g, respectively. The 30 mg/kg treatment also had an effect of increasing the concentration of

carbohydrates and proline, as they were recorded at 12.23±0.56 mg/kg and 6.73±0.28 µmol/g, respectively.

Photosynthetic pigments content

The results in Table 4 show the effect of PAHs treatment at a concentration of 60 mg/kg on the photosynthetic pigments (chlorophyll a and b, and carotenoids). There is a significant decrease in these pigments compared to the comparison sample. The concentration of chlorophyll a and a was 2.47±0.58 and 2.13±1.02 mg/g, respectively. At the concentration of 30 mg/kg of PAHs, there is no significant difference compared to the concentration of 60 mg/kg. However, at the concentration of 10 mg/kg, the content of chlorophyll a and b increased, reaching 3.01±0.58 and 2.01±0.75 mg/g, respectively.

The same trend is observed for carotenoids. The concentration in palm leaves decreases at the highest treatment of PAHs compared to the comparison sample. The decrease is 1.47 ±0.53 mg/g, while the comparison sample is 1.78 ±0.01 mg/g. However, as the concentration of the pollutant decreases, the content of carotenoids increases. When treated at 30 mg/kg of PAHs, the concentration of carotenoids is 1.72±0.43 mg/g, and at a concentration of 10 mg/kg, it reaches 1.79±0.13 mg/g.

Assay of antioxidant enzymes activity

The results of the study (Table 4) showed a statistically significant increase in the activity of antioxidant enzymes at a concentration of 60 mg/kg of PAHs. The activity of enzymes SOD, CAT, and POX was 48±1.83, 6.53±1.54, and 5.42±0.59 units/min, respectively, compared to the control sample, which had activity levels of 2.23±1.005, 4.32±0.56, and 29±1.00 units/min, respectively. At a concentration of 30 mg/kg of PAHs, the activity of antioxidant enzymes (SOD, CAT, and POX) increased to 4.52±0.40, 5.34±1.08, and 41±1.01 units/min, respectively, compared to the control sample.

Regarding the content of MDA and MSI, the results showed that treatment with 60 mg/kg PAHs led to an increase in the MDA content of leaves from 1.32 nmol/g in the control to 4.73 nmol/g in the treatment. This treatment also resulted in a decrease in the MSI to 44%

in the treatment compared to 70% in the control. As shown in Table 3, as the concentration of PAHs in the treatment decreased, the MDA content decreased, but the MSI increased.

Table 3. The effect of different concentrations of PAHs on some biochemical indices of palm leaves

	Naphthalene (mg/kg)	**Anthracene (mg/kg)**	**B(a)p (mg/kg)**	**Proline (µmol/g)**	**Carbohydrate (mg/g)**	**MDA (nmol/g)**	**MSI (%)**
Control	1.60±0.05^{c}	1.30±0.11d	2.63±0.82^{c}	2.64±0.57^{d}	9.88±1.05^{c}	1.32±0.58^{d}	70±8^{a}
Concentration of 60 mg/kg (PAHs)	5.65±0.97^{a}	8.44±1.00^{a}	8.48±0.97^{a}	8.32±0.99^{a}	14.38±0.57^{a}	4.73±0.31^{a}	44±1^{d}
Concentration of 30 mg/kg (PAHs)	3.26±0.95^{b}	5.54±1.00^{b}	6.34±1.00^{b}	6.73±0.28^{b}	12.23±0.56^{b}	3.88±0.82^{b}	50±8^{c}
Concentration of 10 mg/kg (PAHs)	2.98±1.00b^{c}	3.88±1.00^{c}	4.32±0.95^{c}	5.14±1.195^{c}	10.13±0.60^{c}	2.25±0.98^{c}	66±3^{b}

Different letters indicate a significant difference of $P \leq 0.05$ of the samples compared to the control.

	Total chlorophyll (mg/g Fw)	**Chl. a (mg/g Fw)**	**Chl. b (mg/g Fw)**	**Carotenoids (mg/g Fw)**	**POX (units. min^{-1})**	**SOD (units. min^{-1})**	**CAT (units. min^{-1})**

Control	6.71±1.07[a]	4.52±0.5[a]	2.21±0.66[a]	1.78±0.01[ab]	29±1.00[d]	2.23±1.005[d]	4.32±0.56[c]
Concentration of 60 mg/kg (PAHs)	4.61±1.17[d]	2.47±0.58[cd]	2.13±1.02[b]	1.47±0.53[d]	48±1.83[a]	5.42±0.59[a]	6.53±1.54[a]
Concentration of 30 mg/kg (PAHs)	4.91±1.12[cd]	2.90±0.55[c]	2.02±0.70[c]	1.72±0.43[bc]	41±1.01[b]	4.52±0.40[b]	5.34±1.08[b]
Concentration of 10 mg/kg (PAHs)	5.03±1.30[bc]	3.01±0.58[bc]	2.01±0.75[c]	1.79±0.13[a]	33±1.52[c]	3.23±0.57[c]	4.88±0.85[bc]

Table 4. The effect of different concentrations of PAHs on some biochemical indices of palm leaves

Different letters indicate a significant difference of $P \leq 0.05$ of the samples compared to the control.

Discussion:

The problem of environmental pollution is considered a concerning global issue due to the increasing rate of industrialization and population growth, which leads to environmental deterioration (Adamu, 2019). Pea plants (*Pisum sativum* L.) treated with different concentrations of naphthalene showed a slight increase in fatty acid peroxides (MDA) (Agoun et al., 2018). Li et al. (2024) demonstrated that tall fescue plants treated with high concentrations of naphthalene exhibited a decrease in chlorophyll content, an increase in H_2O_2 and MDA levels, and an increase in Djenkolic acid. These findings indicate that plants are exposed to abiotic stresses when exposed to high levels of naphthalene.

Regarding the treatment of plants with the pollutant anthracene, it had a significant effect, particularly at high concentrations. A study by Tomar et al. (2015) showed a decrease in the efficiency of photosynthesis Photosystem II (PSII) during soybean (*Glycine max*) germination due to the toxic effects of anthracene and damage to the oxygen-evolving complex. The secondary plastoquinone reduction centers were converted to non-reducing centers. Spinedi et al. (2021) found that the *Marchantia polymorpha* L plant tolerates anthracene stress at low concentrations but undergoes physiological changes under higher concentrations, including the accumulation of anthracene in tissues. *M. polymorpha* under

PAHs stress conditions, activates two complementary physiological responses: the activation of antioxidant mechanisms and the accumulation of pollutants within plant tissues to mitigate damage to the photosynthetic system.

High molecular weight b(a)p causes cancer (Voloshina et al., 2023). B(a)p is a lipophilic substance that penetrates plant tissues and resists biodegradation. Therefore, it is considered the most stable compound and accumulates throughout the food chain (Fedorenko et al., 2018). It is possible for b(a)p and 1, 2-benzacenaphthene to permeate the metabolic chains of plants by binding to glucose and glutathione (GSH) and entering the cell wall space or cell organelles, causing damage to the infrastructure, and provoking an increase in the intensity of oxidative stress (González, Espinoza et al. 2020). The toxic effect of b(a)p on plants is represented in physiological and biological processes, disrupting cellular structure, reducing chlorophyll, and activating peroxidase and the glutathione chain (Sam, 2022). Voloshina et al. (2023) demonstrated in their experiments on tomato plants treated with b(a)p that physiological and biochemical response indicators were strong, and the results showed a significant increase in MDA and antioxidant enzymes (SOD, POX, and APX). Furthermore, Fedorenko et al. (2021) reported that the accumulation of high levels of b(a)p in barley plants resulted in damage to the plant's tissue and cellular levels, leading to excessive activation of ROS.

To understand the extent of the effect of PAHs on plants, it is important to understand the nature of stress genes and their impact on various biochemical traits. This understanding is crucial for mitigating the negative effects of stress. Plant genes associated with stress and resistance to environmental stresses are connected to several key aspects, including metabolism, cell defense, energy production, cell division and growth, hormone regulation, and other vital functions necessary for plant survival and growth under stressful conditions (Zhang et al., 2021). Multiple types of *STK* genes, along with other plant immunity genes, offer defense mechanisms against pathogens and stresses. For example, studies have shown that *STK* genes like Glutathione S-enzymes (GST) transferase can eliminate cellular toxins generated due to drought stress in *Tamarix hispida* (Yang et al., 2014). Similarly, other research has demonstrated that overexpression of the *STK* genes enhances rice's tolerance to salt (Zhou et al., 2023)

Han et al. (2023) indicated that an increase in gene expression for this gene is consistent with the findings of (Shin et al., 2014), in which PR1 genes are used as markers of systemic acquired resistance (SAR) by stimulating apoptosis. Therefore, it ensures the reduction of pathogen growth in the event of fungal infestation or other stresses associated with hydrocarbon pollution and stress conditions (Akbudak et al., 2020). Activating this system leads to the stimulation of various proteins, including PRs, which are present in all plants. Increasing their expression genetically has proven to enhance the plant's resistance to biotic and abiotic stress conditions (Yuan et al., 2021). Overexpression of the PR1 gene in transgenic tobacco plants enhanced drought and salt tolerance (Sharma et al., 2021).

Stresses that affect water balance within plant cells are among the most common stresses on plants. They impact ion transport, transpiration processes, and more. Therefore, plants activate appropriate mechanisms to confront these environmental stresses, which negatively impact the plant (Berny et al., 2016) and are closely related to photosynthesis, nitrogen fixation, and nutrient absorption (Sun et al., 2015). A previous study demonstrated that barley plants were sensitive to overexpression of the *PIP2* gene, resulting in decreased stress tolerance (Aharon et al., 2003). However, overexpression in rice and tomatoes showed enhanced tolerance under drought and salt stress. One of the mechanisms followed by plants, including palm trees, to maintain the level of photosynthesis is to reduce water-carrying proteins and increase the expression of low-molecular-weight proteins. A study indicated the presence of *PIP2* genes belonging to the AQPS family in the palm genome, encoding proteins with low molecular weight. The overexpression of these genes is found in the leaves, roots, and seeds of palm trees growing under stressful conditions (Patankar et al., 2019).

The ROS content of plant cells is low in natural conditions and does not affect plant cells. However, when plants are exposed to stress, the amount of ROS begins to increase. The first step plants take to reduce the harmful effects of oxygen radicals is to direct the SOD enzyme to convert O^{2-} into a water molecule and hydrogen peroxide (Su et al., 2021). The genes in this family play an important role in transmitting the electron signal and photosynthesis (Huo et al., 2022). They are involved in the plant's resistance to various stress conditions, such as salinity, drought, and pollution.

The concentration of carbohydrates in palm leaves increased in the 60 mg/kg treatment compared to the other treatments. In this treatment, the concentration of carbohydrates reached 14.38 mg/kg, which is higher compared to the control sample. The increase in carbohydrate concentration is attributed to the cumulative effect of PAHs, which stimulates the accumulation of carbohydrates. This accumulation occurs because starch decomposes to provide energy and develop antioxidant energy systems. Plants use this strategy to cope with stressful conditions and stress factors. Abass et al. (2016) also observed an increase in carbohydrate accumulation in palm leaves exposed to salinity stress. Carbohydrates play an important role in cell growth and serve as precursors for the synthesis of free proline. Therefore, they tend to accumulate in plants when exposed to various stresses (Kumar et al., 2017). One possible reason for carbohydrate accumulation is the inhibition of carbohydrate transport from the source (leaves), which reduces their use for growth. Numerous studies have reported the accumulation of carbohydrates in plant tissues exposed to abiotic stresses (Jiang et al., 2017).

The results of the current study indicate an increase in the concentration of the amino acid proline when the plant is treated with PAHs at a concentration of 60 mg/kg compared to the rest of the treatments. Proline helps membrane stability and eliminates ROS under normal conditions. However, when plants are exposed to stress, the production of free radicals exceeds the capacity of antioxidants to counteract them, resulting in oxidative stress. In response to these stresses, plant cells accumulate a large amount of proline (Spormann et al., 2023). Proline is a low molecular weight substance that is dissolved in cell juice.

In our study, the addition of PAHs to the plant induced stress, causing an increase in proline concentration. Proline is an important defense mechanism used by plants to cope with stress by maintaining osmotic balance and neutralizing the negative effects of ROS. It also protects and stabilizes cell membranes, supports photosynthesis and electron transfer processes, and forms strong hydrogen bonds between water and proteins. These functions help preserve membrane proteins, prevent electrolyte leakage, and maintain cell integrity despite stressful conditions and the withdrawal of harmful ions (Ledger et al., 2016).

The study results demonstrate that chlorophyll concentration in palm leaves decreases due to PAHs stress. These findings align with multiple studies that have also observed a decrease in chlorophyll concentration due to aromatic hydrocarbons stress (Sharma et al., 2012; Agoun-Bahar et al., 2018; Sivaram et al., 2020; Li et al., 2024). The decrease in chlorophyll content, particularly at high concentrations, may be attributed to the impact of aromatic hydrocarbons accumulated in high concentrations on the activity of the Rubisco carboxyl group. This inhibition affects the process of photosynthesis, as well as impacts photosystem II, electron flow, and chlorophyll synthesis (Sivaram et al., 2020). Additionally, a significant accumulation of aromatic compounds in cellular membranes can hinder photosynthesis activity and result in the production of a substantial amount of ROS. The study also revealed a decrease in carotenoid pigment levels below a concentration of 60 mg/kg, as findings from other studies examining the effects of PAHs stress on different plants, such as wheat (Shen et al., 2019), *Amaranthus cruentus* (Tandey et al., 2020), and palm (Faisal et al., 2020).

The results demonstrated an increase in enzymes activity when treated with a concentration of 60 mg/kg as well as other treatments, in comparison to the control sample.

When a plant is exposed to stress, it employs a mechanism of enzymatic defense represented by enzymes like SOD, CAT, and POX, to protect cells from the effects of oxidative stress. Our current study reveals an elevation in the concentration of these enzymes, indicating that the PAHs treatment was stressful for the plant. The first step the plant takes to counteract the impact of free radicals is to activate the SOD enzyme, which is considered the primary defense against free radicals. This enzyme converts (O^{2-}) into water and H_2O_2. A previous study conducted by (Alagoz et al., 2023) demonstrated an augmented activity of the SOD enzyme in the leaves of Nursi date palm under salt stress of 150 mM.

The increase in CAT activity can be attributed to its role as the second stage of the enzymatic defense system. It converts H_2O_2 received from the SOD enzyme into water and oxygen, thereby protecting plant cells and tissues from the toxic effects of H_2O_2. The accumulation of proline may contribute to the increase in CAT activity by preventing its denaturation. Our results align with previous studies demonstrating a correlation between

higher cellular proline content and increased CAT enzyme activity in plant cells (Naderi, et al., 2013).

The activity of the POX enzyme also increased when treated with PAHs, whether at low or high concentrations, compared to untreated plants. An increase in POX activity is a vital indicator of plant stress and the formation of ROS. POX helps accumulate other enzymes to reduce the harmful effects of free radicals and protect membrane lipids from oxidation. Its basic function is to convert H_2O_2 into water and oxygen molecules (Molina and Segura, 2021). In a previous study conducted by Shareef (2016), it was found that there was an increase in POX activity in the leaves of Barhi palm seedlings when grown under heavy metal stress.

MDA is an organic compound with the chemical formula $CH_2(COH)_2$. It is formed when fats present in cell membranes are oxidized by ROS. MDA is harmful to the membranes and impacts the activity of enzymes and ions within them. It serves as an indicator of fat oxidation in membranes (Aldesuquy and Ghanem, 2015).

In our study, the concentration of MDA increased significantly when treated with 60 mg per plant compared to other concentrations and the control sample. This suggests that treatment with high concentrations of aromatic hydrocarbons was stressful for the plants, leading to the formation of free radicals and affecting membrane lipids through oxidation. This resulted in the formation of the toxic and potent MDA compound. Alongside the increase in MDA concentration, the membrane stability index decreased when treated with 60 mg, indicating membrane damage at this concentration. However, when treated with 30 mg and 10 mg, the MDA concentration decreased and the membrane stability index increased, indicating a decrease in membrane damage due to oxidation. This finding aligns with the study conducted by Zouari et al. (2016 a, b). MSI is also used as an indicator of membrane safety against stress. A decrease in the MSI value indicates membrane damage (Howladar, 2014).

Conclusion:

The study concludes that the highest PAHs combination led to an increase in the content of aromatic hydrocarbons in the plant, which caused a biochemical stress reflected in an increase in the concentration of carbohydrates and proline and a decrease in its chlorophyll and carotenoid content. Additionally, the activity of antioxidant enzymes (SOD, CAT, and POX) increased, and the lipid peroxidation process occurred through an increase in the content of the compounds MDA and MSI. Moreover, the level of gene expression of the target genes increased. This indicates that the gene expression process of the target genes can be relied upon to predict the occurrence of aromatic hydrocarbon pollution. It can also be concluded that PAHs stress affects many biochemical processes in the plant, and further studies are required to address the causes of aromatic hydrocarbon emissions associated with oil extraction processes.

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