Assessment of oxidative stress response genes in *Avicennia marina* exposed to oil contamination – Polyphenol oxidase *(PPOA)* as a biomarker

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ARTICLE INFO

ABSTRACT

Mangrove plants, which inhabit and form sensitive ecosystems in the intertidal zones of tropical and subtropical coastlines, though vulnerable to petroleum pollution, still maintain their growth under oil contamination. To elucidate the molecular response of mangrove plants to crude oil–sediment mixture, seeds of *Avicennia marina* were planted and grown on 0, 2.5, 5.0, 7.5 and 10 % (w/w) oil-contaminated soil. Plant biomass was highly affected from 3.05 ± 0.28 (Control) to $0.50 \pm .07$ (10 %) and from 3.47 ± 0.12 to 1.88 ± 0.08 in 2 and 4 months old plants respectively. The expression analysis of 11 genes belonging to detoxification pathways in the roots and leaves of 2 and 4 month-old plants was evaluated by qRT-PCR. Our results showed changes in expression levels of *Fe-SOD*, *Mn-SOD*, *CAT*, *PRX*, *PPOs*, *GSTs*, and *NAP*2 whose products are involved in reactive oxygen species (ROS) and xenobiotic detoxification. *PPOA* showed the highest expression induction of 43 ± 1.15, followed by *CAT* (12.61 ± 3.25) and *PPOB* (6.38 ± 1.34) in leaves of 2 months old seedlings grown on 7.5, 10 and 7.5 % oil contaminated soil respectively. *PPOA* (39.23 ± 2.1), *PRX* (32.13 ± 1.2) as well as *PPOB* (26.11 ± 1.3) showed the highest expression induction in leaves of 4 months old plants grown in 2.5 % oil contaminated soil. Our data indicated that PPOA can be a good biomarker candidate gene for long term exposure to oil contamination in *A. marina*.
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1. Introduction

Mangrove ecosystems are essentially vital and adaptable coastline environments, which house a wide diversity of organisms. They stabilize coastlines through their branches and roots which also trap excess deposits, filter water to a better quality favorable for the growth of coral reefs, and greatly reduce coastal erosion by dissipating the energy from incoming waves [1]. The Iranian mangrove forests, which are primarily comprised of *Avicennia marina*, are located in coastal zones of the Persian Gulf and the Gulf of Oman over a range of 1830 km from east to west in southern Iran [2].

Mangrove ecosystems throughout the world face several threats, including pollution, deforestation, fragmentation, and

sea-level rise [3]. These ecosystems are highly vulnerable to oil spills and show a range of stress responses and even lethal effects following oil exposure [4]. Bioindicator species are known as species whose function, population, or status can reveal the qualitative status of the environment. *A. marina* as a prevalent plant species of the Persian Gulf mangrove ecosystems can be used as a bioindicator for environmental pollution in the coastal environment. Biological monitoring of natural ecosystems can be performed by the use of biomarkers from bioindicator species to quantify the degree of exposure to contaminants [5]. Biomarkers are very important in different fields of biological science [6,7] and different biochemical and genetic methods are used to find the best biomarkers [8,6]. A pollution responsive biomarker is a quantitative measure of changes in molecular or cellular components, processes, structures and functions related to exposure to environmental chemicals [9]. Results of the current study can help finding a biomarker gene for oil contamination.
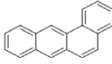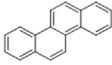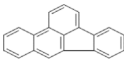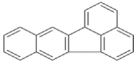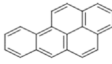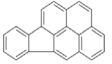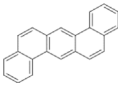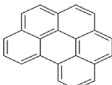
Oil is a complex mixture of different toxic and harmful chemicals that consists of a variety of hydrocarbon-based substances such as

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alkanes, cyclo-hexanes, and polycyclic aromatic hydrocarbons (PAHs) [10]. Research on the physiological and biochemical

Table 1
Effect of 10 % (w/w) oil contamination on the content of polycyclic aromatic hydrocarbons (PAHs) in soil (ng/g).

PAH	Structure	formula	Control	10 % (w/w) oil-contaminated soil
Naphthalene		$C_{10}H_8$	139.97 ± 3.73	6011.2 ± 6.7
Acenaphthylene		$C_{12}H_8$	246.42 ± 3.47	2098.0 ± 1.7
Acenaphthene		$C_{12}H_{10}$	204.13 ± 3.94	1123.0 ± 6.8
Fluorene		$C_{13}H_{10}$	219.59 ± 2.21	2830.0 ± 24.5
Phenanthrene		$C_{14}H_{10}$	75.31 ± 2.23	4978.7 ± 20.6
Anthracene		$C_{14}H_{10}$	ND	120.0 ± 5.3
Fluoranthene		$C_{16}H_{10}$	3.34 ± 0.25	4300.0 ± 10.0
Pyrene		$C_{16}H_{10}$	34.19 ± 1.22	650.0 ± 11.0
Benzo(a)anthracene or Tetraphene		$C_{18}H_{12}$	ND	3691.0 ± 5.5
Chrysene		$C_{18}H_{12}$	ND	150.0 ± 11
Benzo(b)fluoranthene		$C_{20}H_{12}$	ND	405.0 ± 16.0
Benzo(k)fluoranthene		$C_{20}H_{12}$	ND	59.0 ± 4.0
Benzo(a)pyrene		$C_{20}H_{12}$	ND	449.9 ± 13.1
Indeno[1,2,3-cd]pyrene		$C_{22}H_{12}$	ND	83.8 ± 5.5
Dibenz(a,h)anthracene		$C_{22}H_{14}$	ND	57.8 ± 3.4
Benzo(g,h,i)perylene		$C_{22}H_{12}$	ND	153.0 ± 5

responses in mangrove plants to oil contamination has been conducted [11,12], while not much attention has been paid to gene regulation. Some studies with oil components have been performed [13,14] and a hypothesis that oil and PAHs contaminations result in oxidative stress in plants has been suggested [15–17]. Additionally, an increase in the activity of scavenging antioxidant enzymes of mangrove plants in response to oil contamination has been reported [18,19]. To assess if these changes are the result of alterations in the expression of genes, we identified relevant genes and studied their regulation in response to oil contamination. This will allow us to better understand the underlying mechanisms and to screen for bio-indicators for environmental pollution in the future. Genes putatively encoding superoxide dismutases (SODs), catalase (CAT), peroxidase (PRX), ascorbate peroxidase (APX), polyphenol oxidases (PPOs), glutathione-S-transferases (GSTs) and non-intrinsic ABC protein 2 (NAP2) were selected as they are an indication of stress responses. To the best of our knowledge this is the first study in which the molecular response of A. marina to oil contamination was investigated. The findings of the current study, in addition to providing a basal knowledge on responses of A. marina genes related to antioxidant enzymes to long-term oil pollution examined the possibility of using them as biomarkers for oil contamination.

2. Material and methods

2.1. Soil properties and crude oil treatment

Soil was collected from the A horizon of Bagho Nursery site in Bandar Abbas, Hormozgan, Iran. The soil pH was 7.9 and its texture was sandy loam. Soil samples were sieved through a 2 mm mesh, and then sterilized at 121 °C for 2 h. Crude oil (Table1), obtained from Tehran Refinery, was added and mixed with soil thoroughly at concentrations of 2.5, 5.0, 7.5, and 10.0 % (w/w). The control treatment consisted of the same soil that was not mixed with crude oil.

2.2. PAHs assessment

Non-treated control soil and 10 % (w/w) oil-spiked-soil were sampled at the start of the experiment and were used for PAH analysis. The extraction method was adapted from MOOPAM 2010 with some modifications. Briefly, three replicates of 2 g of freeze-dried soil sample were extracted with dichloromethane: acetone (1:1) under sonication, and the solvent was reduced under vacuum with a rotary evaporator. The extract was cleaned up on activated copper for sulfur removal and a silica-alumina column with hexane and hexane-dichloromethane (90:10) as eluents. After removal of

the solvent, the final residue was dissolved in 1 mL hexane. Analysis of PAH was performed with an Agilent 6890 N GC system equipped with a 5973 mass detector and an MSD Chemstation software on an HP-5 fused silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm).

2.3. Plant growth conditions

Mature and uniform propagules of Avicennia marina were collected from Tāsbar Creek of Bandar Abbās-Hormozgan, surface sterilized with 1 % sodium hypochlorite in water for 10 min, and washed thoroughly in sterilized distilled water. Two healthy propagules were sown equidistantly in pots. A total of 25 pots (i.e. 50 plants) were used for each of the five treatments (control and four oil concentrations). Plants were irrigated with 100 mL of water every alternate day. All experiments were carried out in a greenhouse under a temperature regime of 21 and 18 °C during the day and night, respectively. Fresh and dry weights of three biological replicates (each consisting of tissue pooled from 10 plants) were determined on 60 and 120 days after planting.

2.4. Quantitative real-time PCR (qPCR) gene expression analysis

At two time points, 60 and 120 days after planting, all roots and leaves tissues were harvested separately from individual plants and frozen immediately in liquid nitrogen. Samples were freeze-dried (freeze-drier model: OPR-FDB-5503, Korea) and tissue from 10 plants was pooled to generate one biological replicate. RNA was isolated using the Spectrum Plant Total RNA kit (Sigma –Aldrich) following the manufacturer's protocol. An on-column DNase treatment was performed using the RNase-Free DNase set (Qiagen). Total RNA was quantified using a NanoDrop NP-1000 spectrophotometer (NanoDrop Technologies). RNA integrity was checked on a 2100 Bioanalyzer (Agilent). All samples had RNA integrity number (RIN) values above 8. cDNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) and diluted 10 times in ddH$_2$O. Quantitative real-time PCR (qPCR) was performed using the Light Cycler 480 SYBR Green I Master (Roche) on a LightCycler96 system (Roche) programmed as follows (1) preincubation at 95 °C for 5 min, (2) 40 cycles of amplification consisting of 95 °C for 10 s, 55 °C for 10 s and 72 °Cfor 10 s, and (3) melting curve analysis by heating from 65 °C to 97 °C with a ramp rate of 2.2 °C/s. Each 20 μL reaction contained 0.5 μM of each of the forward and reverse primer (Table 2). When possible, primers were designed on annotated Avicennia marina sequences. Otherwise, primers were designed on Avicennia marina sequences that were identified by BLAST with annotated Arabidopsis thaliana

Table 2
Primers used for qRT-PCR of reference and target genes.

	Accession number (where available)	Gene	Product	Forward primers(5′- 3′)	Reverse primers (5′- 3′)
Target Genes	EU025130.1	APX1	Ascorbate peroxidase	GCAATACTGGTGACAAAGTGC	TCGTACAAGTAACTCAGGATCACC
	AY272049.1	CAT	Catalase	ATGGGTCGACGCTTTATCTG	TTGTCGGCCTTACATTGAGG
	AF328859.1	Cu/Zn-SOD	Cu/Zn-Superoxide dismutase	AGGACCACATTCCATAGTTGG	GAAGACCAATGATACCACAAGC
		Fe-SOD	Fe-Superoxide dismutase	CTGGGATTATTCTCCGCTGC	CATCCCAAGAAACAAGATTCTCC
	AY137205.1	Mn-SOD	Mn- Superoxide dismutase	GCCTTTGCTTGGTATTGATGTC	CATAAACTTCACTGGCGTATTTCC
		PPOA	Polyphenol Oxidase	AAGTCCACAACTCCTGGCTG	CCCAGGATTCTTTCGAAGAAG
		PPOB	Polyphenol Oxidase	GGCTTTTCTTTCCCTTCCAC	GCGAAAGTGGGGTCATTTATC
	AB049589.1	PRX	Peroxidase	CAACTAGCCACGGACAAGAGG	TCTCGGACAGAACGGTGATG
		GSTU4	Glutathione-S-Transferase	GAAGGTGCCTGTTCTTGTGC	GCTTTCTCGTACGGATCTTGG
		GSTU25	Glutathione-S-Transferase	TGGAGACAAGACTTACTTTGGAGG	TGCTGAAGTTGCCAAAAGTCTC
		NAP2	Non-intrinsic ABC protein	TTGATGGACTGGAGTCTTGG	GCCAAGATTCAACAACAGATAGC
Reference Genes		ACT2	Actin 2	GTGTGATGTGGATATCAGGAAGG	CCTTAATCTTCATGCTGCTT
		PP2AA3	Protein phosphatase	GCAAATTCTACCCTGTGTAAAGG	CTCAATTGTTGCATCCTTCC
		TIP41-like		AGATGAGTTGGCTGACAATGG	ACTCCATCAACTCTGAGCCAG
		UBQ10	polyubiquitin 10	GCAAGACCATCACTCTCGA	GCTTTCCAGCGAAGATCAGC

Table 3
Changes in fresh and dry weight (in g) of *A. marina* after exposure to different concentrations of crude oil (n≥10 seedlings).

			Control	2.5 %	5.0 %	7.5 %	10.0 %
2 months old plants(g)	**Fresh Weight**	**Root**	1.56 ± 0.23a	1.97 ± 0.36a	1.68 ± 0.44ab	1.14 ± 0.45c	0.41 ± 0.1d
		Shoot	1.49 ± 0.33a	0.88 ± 0.11b	0.69 ± 0.34bc	0.55 ± 0.13c	0.09 ± .05d
		Leaf	0.95 ± 0.24a	0.42 ± 0.13b	0.48 ± 0.11b	0.15 ± 0.04c	0.12 ± 0.03c
		Shoot/Root	1.54 ± 0.28a	0.69 ± 0.2b	0.67 ± 0.29b	0.64 ± 0.12b	0.47 ± 0.12b
	Dry Weight	**Root**	0.27 ± 0.06bc	0.35 ± 0.06a	0.32 ± 0.06ab	0.23 ± 0.09c	0.10 ± 0.07d
		Shoot	0.34 ± 0.07a	0.19 ± 0.02b	0.13 ± 0.03c	0.10 ± 0.03c	0.02 ± 0.01d
		Leaf	0.2 ± 0.01a	0.1 ± 0.01bc	0.12 ± 0.02b	0.04 ± 0.04c	0.04 ± 0.04c
		Shoot/Root	2.00 ± 0.15a	0.82 ± 0.24b	0.76 ± 0.1b	0.62 ± 0.2b	0.59 ± 0.1b
4 months old plants(g)	**Fresh Weight**	**Root**	1.76 ± 0.07bc	2.22 ± 0.11a	1.99 ± 0.19ab	1.41 ± 0.12 cd	1.21 ± 0.10d
		Shoot	1.71 ± 0.17a	1.06 ± 0.07b	0.8 ± 0.07bc	0.62 ± 0.07c	0.59 ± 0.06c
		Leaf	1.46 ± 0.12a	0.61 ± 0.10b	0.57 ± 0.10bc	0.34 ± 0.05 cd	0.28 ± 0.05d
		Shoot/Root	1.89 ± 0.15a	0.77 ± 0.14b	0.70 ± 0.1b	0.69 ± 0.12b	0.74 ± 0.11b
	Dry Weight	**Root**	0.29 ± 0.04b	0.38 ± 0.08a	0.37 ± 0.1a	0.28 ± 0.07b	0.24 ± 0.06b
		Shoot	0.38 ± 0.11a	0.2 ± 0.09b	0.15 ± .04bc	0.11 ± .04c	0.1 ± 0.03c
		Leaf	0.3 ± 0.08a	0.13 ± .08b	0.13 ± 0.07b	0.08 ± .04c	0.07 ± 0.04c
		Shoot/Root	2.36 ± 0.1a	0.87 ± 0.27b	0.78 ± 0.24b	0.67 ± 0.28b	0.71 ± 0.2b

Values in each line marked with the same letter do not differ significantly at $p \leq 0.05$.

genes on the gene databases and sequence read archive (SRA) of NCBI (https://www.ncbi.nlm.nih.gov) and the Mangrove Transcriptome Database (http://mangrove.illinois.edu/transcriptome). Quantification cycle (Cq) values for each amplification curve were determined by the LightCycler 96 software version 1.1 (Roche). LinRegPCR software [20,21] was used to determine the mean PCR efficiency for each primer pair. After analyzing the stability of the genes selected as possible reference genes by geNorm [22] in qbase + *ACT2*, *UBQ10*, and *TIP41-like* were chosen as reference genes (while excluding *PP2AA3*) providing a more accurate normalization compared to the use of a single non-validated reference gene.

2.5. Statistical analyses

Statistical analysis of the effect of oil on morphological variables and gene expression ratios in leaves and roots of oil treated samples compared to control samples was performed with Graphpad Prism v.8 (GraphPad, USA) and qbase + version2.6.1 [23] respectively. Heatmap correlation analysis was performed using MetaboAnalyst web portal (https://www.metaboanalyst.ca/). Principal component analysis (PCA) was conducted using publicly available Past3.16 software.

3. Results

3.1. Effect of oil contamination on fresh and dry weight of Avicennia marina

The contamination of soil with crude oil caused an increase in the total PAHs in soil samples at the beginning of the experiment (Table 1). In soil polluted with 10 % oil, the sum of PAHs increased about 30-fold relative to the control (not contaminated). Among the PAHs, two-ringed naphthalene showed the highest increase with oil contamination.

The fresh and dry weight of *A. marina* seedlings decreased significantly under oil contamination (Table 3). Plant seedlings showed significantly greater root biomass in the 2.5 and 5 % w/w soil contaminated treatments as compared with the control over the growth period. Leaf biomass decreased significantly in all treatments as compared with the control. In the presence of oil, the shoot/root ratio changed in favor of greater root production. Some plants exhibited a lack of shoot initiation and growth at 10 % oil contamination as they developed roots but not shoots.

3.2. Gene expression changes in Avicennia marina in response to oil exposure

Plants induced specific gene responses, depending on the treatment they were exposed to. The expression patterns of 11 genes: *Mn-SOD*, *Fe-SOD*, *Cu/Zn-SOD*, *CAT*, *PPOA*, *PPOB*, *APX1*, *PRX*, *GSTU4*, *GSTU25* and *NAP2*, which belong to antioxidative and detoxification pathways were assayed by qPCR in leaf and root tissues of 2 and 4 months old *A. marina* grown on soil contaminated with different concentrations of oil. geNorm analysis on the stability of putative reference gene expression revealed that *ACT2*, *UBQ10* and *TIP41-like* were suitable reference genes for the assessment of antioxidative enzyme gene expression of *A. marina* in response to oil contamination.

3.3. Gene expression changes in leaves of plants exposed to oil

In leaves of 2 months old plants two of the 11 selected genes, *PPOA* and *CAT* were induced significantly ($p < 0.05$) by all treatments compared with control plants (Fig. 1). The expression of five other genes: *PPOB*, *Mn-SOD*, *Fe-SOD*, *PRX* and *GSTU4* showed a significant induction by at least one of the treatments, with 7.5 % oil affecting most of them. No significant differences in gene expression were observed in leaves for *Cu/Zn-SOD*, *GSTU25* and *NAP2* at any of the assayed concentrations of crude oil. *GSTU4* was the only gene whose transcript level was significantly reduced by oil exposure in two months old leaves (Fig. 1).

After 4 months of oil exposure, 8 of the 11 selected genes were significantly ($p < 0.05$) induced in leaves by all treatments compared with control plants (Fig. 1), with *PPOA*, *PPOB* and *PRX* showing particularly high induction levels. In addition, the expression of *CAT*, *Cu/Zn-SOD*, and *APX1* showed a significant induction by the 2.5 % oil treatment.

3.4. Gene expression changes in roots of plants exposed to oil

In the roots of two months old plants, the expression of *GSTU25* was significantly reduced in all treatments (Fig. 2). Four other genes (*GSTU4*, *PPOA*, *Mn-SOD*, *NAP2*) were repressed by at least one of the oil treatments, most by the 5 % oil exposure. No significant differences were observed in gene expression of *CAT*, *APX1* and *Cu/Zn-SOD* under any of the treatments. Only a few genes were significantly induced in two months old roots, such as *PPOB*, *PRX*
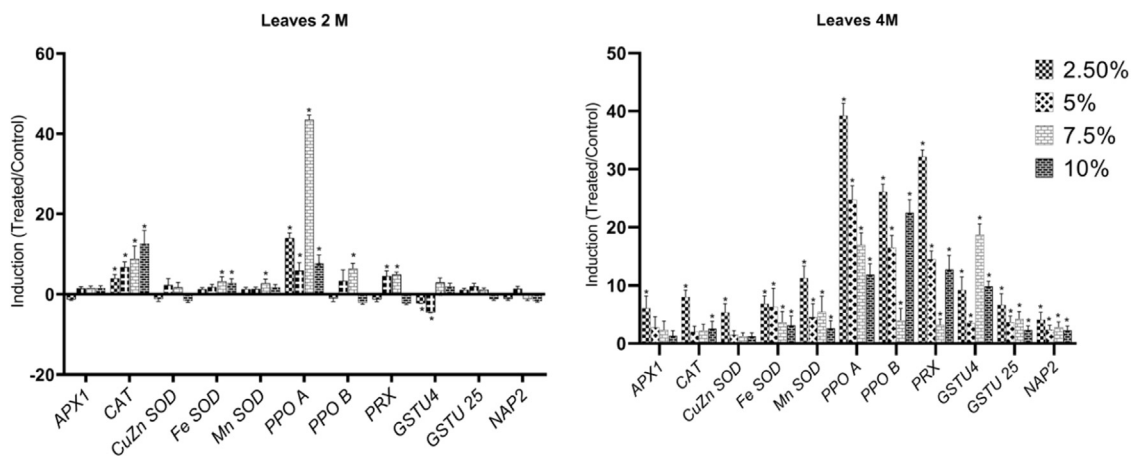
Fig. 1. Changes in transcriptional level of selected genes in leaves of two and four months old *A. marina* exposed to different levels of oil contamination. Means SEs, n = 3, *-significant differences between the control and treated samples ($P \leq 0.05$; *t-test*).

Fig. 2. Changes in transcriptional level of selected genes in roots of two and four months old *A. marina* exposed to different levels of oil contamination. Means SEs, n = 3, *-significant differences between the control and treated samples ($P \leq 0.05$; *t-test*).
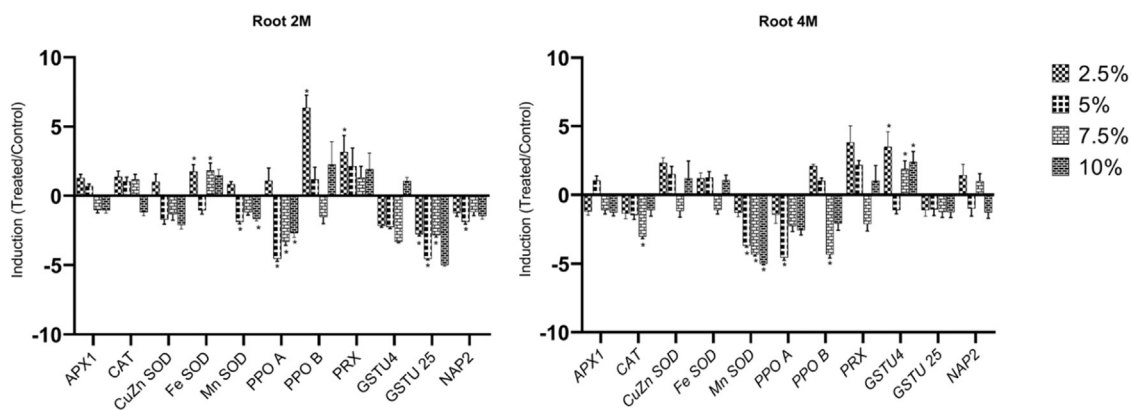
and *Fe-SOD,* which were induced 6.37, 3.16 and 1.75 fold, respectively by the 2.5 % oil treatment

Among the 11 genes that were assessed, the six genes *PRX*, *GSTU25*, *APX1*, *Fe-SOD*, *NAP2,* and *Cu/Zn-SOD* were not significantly affected by any of the oil treatments in roots of 4 months old plants (Fig. 2). The expression of four genes was reduced under at least one treatment: *Mn-SOD* under 5 %, 7.5 % and 10 %, *PPOB* and *CAT* under 7.5 % and PPOA under 5 % oil contamination. Only the expression of *GSTU4* was significantly induced in the roots of four months old plants exposed to oil (Fig. 2).

Heatmap and PCA analysis of the induction level of the selected genes of *A. marina* seedlings grown on oil contaminated soils showed that under the four levels of oil concentration, leaves and roots showed a completely different response (Fig. 3). Changes in transcript levels of *PRX* and *PPOB* (Pearson correlation coefficient or PCC = 0.92), Mn-SOD and *Fe-SOD* (PCC = 0.87), and *APX* and *Mn-SOD* (PCC = 0.86) showed a strong correlation under oil contamination.

The PCA of transcriptional changes of the selected genes showed that about 90 % of variation between treatments could be explained by two principal components (Fig. 3b). The first component (PCA1) separated the four months old leaves from other samples primarily based on PPOA, PPOB and PRX expressional changes (Fig. 3b) (x-axis). Treated two and four months old root samples completely separated from the leaves by PC1. The second component (PCA2) separated mostly two months old leaves samples from the other samples, which was mainly based on PPOA, PPOB and PRX expression levels (y-axis).

4. Discussion

Petroleum contamination of the rhizosphere affects plant functions both physically by attaching to roots and through dissipation of volatile compounds. Coating blockage of crude oil on roots surface may cause low water accessibility and oxygen deficiency [24]. Under water deficiency, plant growth is readily inhibited and growth of roots is favored over that of leaves [25]. Previous reports showed that the presence of petroleum hydro-carbons can be toxic and significantly reduce plant biomass [26–28]. In our study, growth inhibition of crude oil on *A. marina* was obvious and similar to those reported by other investigators [14,29].

Our study confirmed that *A. marina* plants tolerate mid-term exposure to mild oil contamination in soil, even though a significant decrease in plant growth was observed at higher concentrations. The enhanced root growth at lower oil concentrations may be due to a stress response [30] or be a strategy for the plant to stimulate water and nutrient uptake [31].

Oil contamination is known to be one of the main abiotic stress types for mangroves and leads to the production of reactive oxygen species (ROS) [18,24]. Reports have documented that oil contamination provokes an increase in cellular levels of ROS, leading to oxidative damage enhancing the stress in plants [18,32,33]. Nonetheless, plant cells have developed different strategies such as enzymatic and non-enzymatic defense systems in order to mitigate oxidative stress [34].
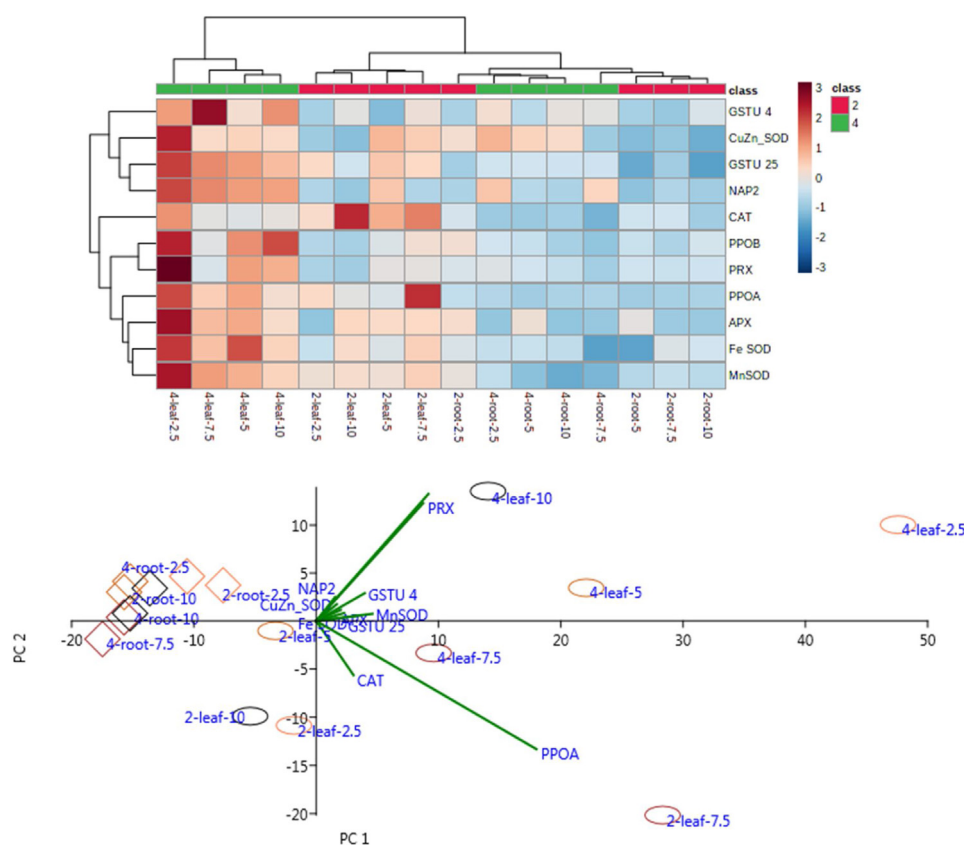
Fig. 3. Heatmap and PCA analysis of the change in transcript levels of the selected genes in two and four months old root and leaf of *A. marina* grown on 2.5 to 10 % (W/W) oil contaminated soil.

The excess ROS in plants seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids, and may eventually cause plant growth inhibition or even death. To respond to the oxidative stress, activities of a series of antioxidative enzymes including different forms of CAT, SOD, and PRXs in plants increase to better scavenge ROS. These changes in enzyme activities can be due to an increase in the expression level of their corresponding genes [35].

We observed overall greater changes in gene expression in leaves compared to roots, which may indicate a higher sensitivity of leaves to the oil contamination stress. Genes were also much more responsive to oil contamination in leaves of four months old plants in comparison with two months old plants, which may be because of the longer exposure to contamination. The nutrients stored in cotyledons might also have a mitigating effect on the stress [36] and this effect would be expected to be stronger in younger plants.

SOD is involved in the first step of ROS elimination by catalyzing the conversion of O_2^- to H_2O_2 and O_2, H_2O_2 being further decomposed by CAT, PRXs and APX [37]. Our findings of increased expression levels of *SODs, PRX* and *APX*, especially in the leaves of four months old plants, under oil treatment are in agreement with observations of Liu et al. [16] who reported an increase in the SOD, PRX and APX enzyme activities in *A. thaliana* under phenanthrene treatment. The observed upregulation of these genes may help plants to reduce the deleterious effects of ROS cytotoxicity. This finding is also consistent with transcriptional studies that reported an increase in *APX1* transcripts following phenanthrene treatment [16]. In our data *CAT* expression was upregulated in leaves, while its expression was not affected or even down-regulated in roots exposed to oil.

Other enzymes such as PPOs are known to catalyze the oxidative transformation of a large number of phenolic and non-phenolic aromatic compounds to their corresponding quinones which are insoluble and less toxic [38,39]. Liu et al. [40] showed for example that the rhizosphere soil PPO activity of *Echinacea purpurea* and *Festuca arundinacea* Schred increased after cultivation on PAH-contaminated soils. The induction of *PPOA* and *PPOB* in leaves of *A. marina* in our study may be related to their physiological function in PAH degradation process [40] and [41].

Proteomic analysis of *A. thaliana* exposed to phenanthrene indicated that antioxidant activity is the most significant term in the molecular function ontology [42]. That study also showed that phenanthrene exposure induced reactive oxygen formation and significantly altered the activities of enzymes such as CAT, APX and peroxiredoxins in *A. thaliana*. Our findings are therefore in agreement with previous physiological, transcriptional, biochemical and proteomics studies in *A. thaliana* which implied oxidative stress as a major component of plant response to PAH contamination [15,16,43,44].

Youssef [14] showed a linear relationship between the PAHs doses applied to *A. marina* seedlings and the amounts accumulated in their leaf tissue. Additionally, Jia et al. [45] reported increased concentrations of phenanthrene and pyrene in *A. marina* leaves with enhancing their sediment concentrations. These observations are consistent with a significant decrease of PAHs concentration in rhizospheric soil of *A. marina* in comparison with a non-rhizospheric control (Moradi et al., under publication), an indication for plant uptake of PAHs in our assays. A transfer of PAHs from root to shoot may be the main cause of gene induction in leaves. Glutathione S-transferases (GSTs) are enzymes that conjugate the reduced form of glutathione (GSH) to xenobiotic

substrates to facilitate their detoxification. They can also function as antioxidants by tagging oxidative degradation products or by acting as a glutathione peroxidase [46–48]. In *A. thaliana GSTU25* was induced upon exposure to oil [17]. *GSTU4* and *GSTU25* were induced by phenanthrene [44] and in roots treated with the organic fraction of oil sands process affected water [49]. Inductions of *GSTU4* and *GSTU25* in leaves of four months old *A. marina* grown on oil contaminated soil may be related to these broad roles of *GSTs*.

Coordinated changes in expression levels of *NAP2* and *GSTU25* in response to oil contamination may be related to their sequential role in the PAHs detoxification process. As the first step of detoxification, members of the cytochrome P450 family catalyze the oxidation of potentially toxic compounds, which are subsequently conjugated to a hydrophilic molecule, such as glucose, glutathione or glucuronide [50,51]. This conjugation step makes the potentially toxic compounds more hydrophilic and prevents the newly formed compounds from crossing membranes by diffusion. As mentioned above, glutathione conjugation of xenobiotics is catalyzed by various GSTs [52]. As the final step, compound-conjugates can be transported into the vacuole or apoplast by ABC transporters. This process further reduces the toxicity of the compounds [53]. In *A. thaliana*, *NAP2* is known as a gene that encodes a member of the *NAP* subfamily of ABC transporters. Upregulation of *NAP2* was reported in *A. thaliana* under exposure to oil and phenanthrene [15,44]. Taken together, our results of the upregulation of *GSTs* and *NAP2* genes in leaves of 4 months old *A. marina* suggest their potential roles in PAHs detoxification.

Oxidative stress-related enzymes, because of their high sensitivity, have been suggested as biomarkers for recognition of the harm induced by contaminants or other environmental stresses in plants [54–56]. The description of the cause–effect relationship is necessary for biomarker validation [55], although such data are still very scarce. Among the eleven genes assayed in the current study, *PPOA* showed a significant and strong (more than fivefold) up-regulation in leaves of 2 and 4 months old seedlings under all tested oil concentrations (Fig. 1). It is therefore a very good candidate for further studies as a biomarker of oil contamination in *A. marina*.

5. Conclusion

Our study provides the basis for the investigation of antioxidative stress responsive genes of *A. marina* to oil contamination. Due to the limited number of genes assayed in the current study further efforts are needed in order to identify robust biomarker genes. Strong induction of the genes in leaves as compared to roots in both 2 and 4 months old plants confirmed that the leaves are a better source to find biomarkers for oil contamination. Our data suggest that *PPOA* could be used as a biomarker for oil contamination in the mangrove ecosystem as its strong induction may be related to its physiological function in the PAH degradation process. Research into the possible use of *PPOA* as biomarker of oil contamination in the mangrove ecosystem of the Persian Gulf and its coastal areas, the world's largest source of petroleum and related industries, is already underway with a particular focus on Nayband Bay in Asaluyeh- the south of Iran.

Author statement

Atle M. Bones and Hassan Zare Maivan supervised the research with the assistance of Mehri Seyed Hashtroudi; Babak Moradi and Mona Sorahinobar carried out experiments with the assistance of Torfinn Sparstad; Babak Moradi, Ralph Kissen and Mona

Sorahinobar analyzed experimental results and data and wrote the draft of manuscript. All authors read and approve the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

References

[1] D.M. Alongi, Present state and future of the world's mangrove forests, Environ. Conserv. 29 (3) (2002) 331–349, doi:http://dx.doi.org/10.1017/S0376892902000231.
[2] S. Rashvand, S.M. Sadeghi, Distribution, characteristics and economic importance of mangrove forests in Iran, Mangrove Ecosystems of Asia, Springer, 2014, pp. 95–126, doi:http://dx.doi.org/10.1007/978-1-4614-8582-7_6.
[3] C. Giri, et al., Status and distribution of mangrove forests of the world using earth observation satellite data, Global Ecol. Biogeogr. 20 (2011) 154–159, doi:http://dx.doi.org/10.1111/j.1466-8238.2010.00584.x.
[4] N.C. Duke, Oil spill impacts on mangroves: recommendations for operational planning and action based on a global review, Mar. Pollut. Bull. 109 (2016) 700–715, doi:http://dx.doi.org/10.1016/j.marpolbul.2016.06.082.
[5] A. Sureda, A. Box, S. Tejada, A. Blanco, J. Caixach, S. Deudero, Biochemical responses of Mytilus galloprovincialis as biomarkers of acute environmental pollution caused by the Don Pedro oil spill (Eivissa Island, Spain), Aquat. Toxicol. 101 (2011) 540–549, doi:http://dx.doi.org/10.1016/j.aquatox.2010.12.011.
[6] M. Al Za'abi, B. H Ali, I. Ali, Advances in the methodologies for the analysis of acute kidney injury biomarkers, Recent Pat. Biomark. 1:5 (2) (2015) 81–92, doi:http://dx.doi.org/10.2174/2210309006666151123190057.
[7] X.S. Yang, J. Wu, T.E. Ziegler, X. Yang, A. Zayed, M.S. Rajani, et al., Gene expression biomarkers provide sensitive indicators of in planta nitrogen status in maize, Plant Physiol. 157 (4) (2011) 1841–1852, doi:http://dx.doi.org/10.1104/pp.111.187898.
[8] I. Ali, Z. A Al-Othman, K. Saleem, A. Hussain, I. Hussain, Role of chromatography for monitoring of breast cancer biomarkers, Recent Pat. Biomark. 1 (1(1)) (2011) 89–97, doi:http://dx.doi.org/10.2174/2210309011101010089.
[9] T. Dalzochio, G.Z.P. Rodrigues, I.E. Petry, G. Gehlen, L.B. da Silva, The use of biomarkers to assess the health of aquatic ecosystems in Brazil: a review, Int. Aquat. Res. 8 (4) (2016) 283–298, doi:http://dx.doi.org/10.1007/s40071-016-0147-9.
[10] W.E. Rudzinski, T.M. Aminabhavi, A review on extraction and identification of crude oil and related products using supercritical fluid technology, Energ Fuel 14 (2000) 464–475, doi:http://dx.doi.org/10.1021/ef990177i.
[11] Y. Yong, N. Tam, Effects of used lubricating oil on two mangroves Aegiceras corniculatum and Avicennia marina, J. Environ. Sci. 19 (2007) 1355–1360, doi:http://dx.doi.org/10.1016/S1001-0742(07)60221-6.
[12] C. Zhang, K. Leung, Y. Wong, N. Tam, Germination, growth and physiological responses of mangrove plant (Bruguiera gymnorrhiza) to lubricating oil pollution, Environ. Exp. Bot. 60 (2007) 127–136.
[13] S. Olubodun, G. Eriyamremu, Effect of different crude oil fractions on growth and oxidative stress parameters of maize radicle, Int. J. Plant Soil Sci. 2 (2013) 144–154, doi:http://dx.doi.org/10.9734/IJPSS/2013/4102.
[14] T. Youssef, Physiological responses of Avicennia marina seedlings to the phytotoxic effects of the water-soluble fraction of light Arabian crude oil, Environmentalist 22 (2002) 149–159, doi:http://dx.doi.org/10.1023/A:1015385700669.
[15] H. Liu, et al., Stress signaling in response to polycyclic aromatic hydrocarbon exposure in Arabidopsis thaliana involves a nucleoside diphosphate kinase, NDPK-3, Planta (2014) 1–13, doi:http://dx.doi.org/10.1007/s00425-014-2161-8.
[16] H. Liu, D. Weisman, Ye Y-b, B. Cui, Y.-h Huang, A. Colón-Carmona, Z.-h Wang, An oxidative stress response to polycyclic aromatic hydrocarbon exposure is rapid and complex in Arabidopsis thaliana, Plant Sci. 176 (2009) 375–382, doi:http://dx.doi.org/10.1016/j.plantsci.2008.12.002.
[17] S.M. Nardeli, et al., Transcriptional responses of Arabidopsis thaliana to oil contamination, Environ. Exp. Bot. 127 (2016) 63–72, doi:http://dx.doi.org/10.1016/j.envexpbot.2016.03.007.
[18] L. Ke, C. Zhang, Y.S. Wong, N.F.Y. Tam, Dose and accumulative effects of spent lubricating oil on four common mangrove plants in South China, Ecotox.

Environ. Safe 74 (2011) 55–66, doi:http://dx.doi.org/10.1016/j.ecoenv.2010.09.011.

[19] V. Sodré, et al., Physiological aspects of mangrove (*Laguncularia racemosa*) grown in microcosms with oil-degrading bacteria and oil contaminated sediment, Environ Pollut 172 (2013) 243–249, doi:http://dx.doi.org/10.1016/j.envpol.2012.09.003.

[20] C. Ramakers, J.M. Ruijter, R.H.L. Deprez, A.F. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, Neurosci. Lett. 339 (2003) 62–66, doi:http://dx.doi.org/10.1016/S0304-3940(02)01423-4.

[21] J. Ruijter, C. Ramakers, W. Hoogaars, Y. Karlen, O. Bakker, M. Van den Hoff, A. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, Nucleic Acids Res. 37 (2009) e45, doi:http://dx.doi.org/10.1093/nar/gkp045.

[22] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (7) (2002), doi:http://dx.doi.org/10.1186/gb-2002-3-7-research0034 research0034-1.

[23] J. Hellemans, G. Mortier, A. De Paepe, F. Speleman, J. Vandesompele, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data, Genome Biol. 8 (1) (2007).

[24] C. Zhang, K. Leung, Y. Wong, N. Tam, Germination, growth and physiological responses of mangrove plant (*Bruguiera gymnorrhiza*) to lubricating oil pollution, Environ. Exp. Bot. 60 (2007) 127–136, doi:http://dx.doi.org/10.1016/j.envexpbot.2006.09.002.

[25] T.C. Hsiao, L.K. Xu, Sensitivity of growth of roots versus leaves to water stress: biophysical analysis and relation to water transport, J. Exp. Bot. 51 (2000) 1595–1616, doi:http://dx.doi.org/10.1093/jexbot/51.350.1595.

[26] P.A. Kulakow, A. Schwab, M. Banks, Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments, Int. J. Phytoremediat. 2 (2000) 297–317, doi:http://dx.doi.org/10.1080/15226510008500040.

[27] D.B. Robson, J.D. Knight, R.E. Farrell, J.J. Germida, Ability of cold-tolerant plants to grow in hydrocarbon-contaminated soil, Int. J. Phytoremediat. 5 (2003) 105–123, doi:http://dx.doi.org/10.1080/713610174.

[28] M. Tesar, T.G. Reichenauer, A. Sessitsch, Bacterial rhizosphere populations of black poplar and herbal plants to be used for phytoremediation of diesel fuel, Soil Biol. Biochem. 34 (2002) 1883–1892, doi:http://dx.doi.org/10.1016/S0038-0717(02)00202-X.

[29] N.F. Tam, T.W. Wong, Y. Wong, A case study on fuel oil contamination in a mangrove swamp in Hong Kong, Mar. Pollut. Bull. 51 (2005) 1092–1100, doi:http://dx.doi.org/10.1016/j.marpolbul.2005.06.005.

[30] N. Merkl, R. Schultze-Kraft, C. Infante, Phytoremediation in the tropics—the effect of crude oil on the growth of tropical plants, Bioremediat. J. 8 (2004) 177–184, doi:http://dx.doi.org/10.1080/10889860490887527.

[31] C. Frick, J. Germida, R. Farrell, Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites, Technical Seminar on Chemical Spills, 1999, Environment Canada, 1998, pp. 105a–124a.

[32] V. Pašková, K. Hilscherová, M. Feldmannová, L. Bláha, Toxic effects and oxidative stress in higher plants exposed to polycyclic aromatic hydrocarbons and their N-heterocyclic derivatives, Environ. Toxicol. Chem. 25 (2006) 3238–3245.

[33] H. Song, Y.-S. Wang, C.-C. Sun, M.-L. Wu, Y.-L. Peng, C. Deng, Q.P. Li, Effects of polycyclic aromatic hydrocarbons exposure on antioxidant system activities and proline content in *Kandelia candel*, Oceanol. Hydrobiol. Stud. 40 (2011) 9–18, doi:http://dx.doi.org/10.2478/s13545-011-0024-5.

[34] S.K. Das, J.K. Patra, H. Thatoi, Antioxidative response to abiotic and biotic stresses in mangrove plants: a review, Int. Rev. Hydrobiol. 101 (1–2) (2016) 3–19, doi:http://dx.doi.org/10.1002/iroh.201401744.

[35] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930, doi:http://dx.doi.org/10.1016/j.plaphy.2010.08.016.

[36] Z.Z. Yan, L. Ke, N.F.Y. Tam, Lead stress in seedlings of *Avicennia marina*, a common mangrove species in South China, with and without cotyledons, Aquat. Bot. 92 (2010) 112–118, doi:http://dx.doi.org/10.1016/j.aquabot.2009.10.014.

[37] K. Apel, H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, Annu. Rev. Plant Biol. 55 (2004) 373–399, doi:http://dx.doi.org/10.1161/CIRCRESAHA.117.311401.

[38] A.M. Mayer, Polyphenol oxidases in plants and fungi: going places? A review, Phytochemistry 67 (21) (2006) 2318–2331, doi:http://dx.doi.org/10.1016/j.phytochem.2006.08.006.

[39] S. Mukherjee, B. Basak, B. Bhunia, A. Dey, B. Mondal, Potential use of polyphenol oxidases (PPO) in the bioremediation of phenolic contaminants containing industrial wastewater, Rev. Environ. Sci. Biotechnol. 12 (2013) (2013) 61–73, doi:http://dx.doi.org/10.1007/s11157-012-9302-y.

[40] R. Liu, Y. Dai, L. Sun, Effect of rhizosphere enzymes on phytoremediation in PAH-contaminated soil using five plant species, PLoS One 10 (2015)e0120369, doi:http://dx.doi.org/10.1371/journal.pone.0120369.

[41] W. Ling, X. Lu, Y. Gao, J. Liu, Y. Sun, Polyphenol oxidase activity in subcellular fractions of tall fescue contaminated by polycyclic aromatic hydrocarbons, J. Environ. Qual. 41 (2012) 807–813, doi:http://dx.doi.org/10.2134/jeq2011.0461.

[42] H. Liu, D. Weisman, L. Tang, et al., Stress signaling in response to polycyclic aromatic hydrocarbon exposure in Arabidopsis thaliana involves a nucleoside diphosphate kinase, NDPK-3, Planta 241 (2015) 95–107, doi:http://dx.doi.org/10.1007/s00425-014-2161-8.

[43] M. Alkio, T.M. Tabuchi, X. Wang, A. Colón-Carmona, Stress responses to polycyclic aromatic hydrocarbons in Arabidopsis include growth inhibition and hypersensitive response-like symptoms, J. Exp. Bot. 56 (2005) 2983–2994, doi:http://dx.doi.org/10.1093/jxb/eri295.

[44] D. Weisman, M. Alkio, A. Colón-Carmona, Transcriptional responses to polycyclic aromatic hydrocarbon-induced stress in *Arabidopsis thaliana* reveal the involvement of hormone and defense signaling pathways, BMC Plant Biol. 10 (2010) 1, doi:http://dx.doi.org/10.1186/1471-2229-10-59.

[45] H. Jia, H. Wang, H. Lu, S. Jiang, M. Dai, J. Liu, C. Yan, Rhizodegradation potential and tolerance of *Avicennia marina* (Forsk.) Vierh in phenanthrene and pyrene contaminated sediments, Mar. Pollut. Bull. 110 (2016) 112–118, doi:http://dx.doi.org/10.1016/j.marpolbul.2016.06.075.

[46] I. Cummins, D.P. Dixon, S. Freitag-Pohl, M. Skipsey, R. Edwards, Multiple roles for plant glutathione transferases in xenobiotic detoxification, Drug Metab. Rev. 43 (2011) 266–280, doi:http://dx.doi.org/10.3109/03602532.2011.552910.

[47] D.A. Dalton, et al., Physiological roles of glutathione S-transferases in soybean root nodules, Plant Physiol. 150 (2009) 521–530, doi:http://dx.doi.org/10.1104/pp.109.136630.

[48] N.E. Labrou, A.C. Papageorgiou, O. Pavli, E. Flemetakis, Plant GSTome: structure and functional role in xenome network and plant stress response, Curr. Opin Biotech. 32 (2015) 186–194, doi:http://dx.doi.org/10.1016/j.copbio.2014.12.024.

[49] E.E. Widdup, K. Chatfield-Reed, D. Henry, G. Chua, M.A. Samuel, D.G. Muench, Identification of detoxification pathways in plants that are regulated in response to treatment with organic compounds isolated from oil sands process-affected water, Chemosphere 139 (2015) 47–53, doi:http://dx.doi.org/10.1016/j.chemosphere.2015.05.048.

[50] J.C. Hernández-Vega, et al., Detoxification of polycyclic aromatic hydrocarbons (PAHs) in *Arabidopsis thaliana* involves a putative flavonol synthase, J. Hazard. Mater. 321 (2017) 268–280, doi:http://dx.doi.org/10.1016/j.jhazmat.2016.08.058.

[51] K. Kreuz, R. Tommasini, E. Martinoia, Old enzymes for a new job (herbicide detoxification in plants), Plant Physiol. 111 (1996) 349, doi:http://dx.doi.org/10.1104/pp.111.2.349.

[52] N. Rouhier, S.D. Lemaire, J.-P. Jacquot, The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation, Annu. Rev. Plant Biol. 59 (2008) 143–166, doi:http://dx.doi.org/10.1146/annurev.arplant.59.032607.092811.

[53] J. Kang, J. Park, H. Choi, B. Burla, T. Kretzschmar, Y. Lee, E. Martinoia, Plant ABC Transporters the Arabidopsis book, (2011), pp. e0153, doi:http://dx.doi.org/10.1199/tab.0153.

[54] M. Tausz, H. Šircelj, D. Grill, The glutathione system as a stress marker in plant ecophysiology: is a stress-response concept valid? J. Exp. Bot. 55 (2004) 1955–1962 ps://doi.org/10.1093/jxb/erh194.

[55] F.F. Caregnato, C.E. Koller, G.R. MacFarlane, J.C. Moreira, The glutathione antioxidant system as a biomarker suite for the assessment of heavy metal exposure and effect in the grey mangrove, *Avicennia marina* (Forsk.) Vierh, Mar. Pollut. Bull. 56 (2008) 1119–1127, doi:http://dx.doi.org/10.1016/j.marpolbul.2008.03.019.

[56] S. Harish, K. Murugan, Oxidative stress indices in natural populations of *Avicennia alba* Blume. as biomarker of environmental pollution, Environ. Res. 111 (2011) 1070–1073, doi:http://dx.doi.org/10.1016/j.envres.2011.07.002.