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ORIGINAL ARTICLE

Induction of Oxidative Stress and Anatomical Changes by Polycyclic Aromatic Hydrocarbons in *Medicago sativa* L.

Leyla Jafari[1], Maryam Khoshsokhan-Mozaffar[*1], Elaheh Vatankhah[2]

[1] Department of Biology, Basic science faculty, Qom Branch, Islamic Azad University, Qom, Iran

[2] Department of Biology, Faculty of Sciences, University of Zanjan, Zanjan, Iran

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KEYWORDS

Anthracene;
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ABSTRACT: In this study effect of anthracene on germination, anatomy and oxidative stress in *Medicagosativa* was evaluated. Seed germination, length and weight of seedlings were measured after seven days of treatment (2 and 4 mmol L^{-1}). After twelve days, anatomical changes and activity of Superoxide Dismutase, Polyphenol Oxidase, Ascorbate Peroxidase, Glutathione Transferase, Soluble Peroxidase, Malondialdehyde in shoots and roots, as well as chlorophyll content of aerial parts, were determined. Also, morphological changes during the growth in complete plants were studied. The results showed that, anthracene had no significant effect on seed germination, but reduced the length of seedlings and the weight of them. The activity of mentioned enzymes in the shoot and often in the roots, in 4 mmol L^{-1} anthracene significantly was increased compared to the control plants. Anthracene treatment decreased significantly Malondialdehyde levels in shoot, while it increased significantly in roots and this treatment had no significant effect on chlorophyll a and b contents. Periderm diameter increased in treated roots and xylem extent reduced in treated shoots. It seems that the low water solubility of anthracene, also, the low sensitivity of alfalfa to PAHs, partially stabilize the plant to low concentration of Anthracene.

INTRODUCTION

By increasing environmental pollution, the study of abiotic stress responses in plants has become ever more important in agriculture, forest management, and ecosystem restoration strategies [1-3].

Organic pollutants accumulate in vegetation [4, 5] and can cause health problems [6, 7]. Some of the studied stresses factors are include: heavy metals, ozone, UV (Ultraviolet) light, salinity, and drought. The polycyclic aromatic hydrocarbons (PAHs) pollutants are poorly studied as stress inducers. PAHs are organic compounds composed of multiple aromatic rings, which make these molecules highly persistent in the environment. Many PAHs in the environment are typically found as complex mixtures. Lower-temperature combustion, such as tobacco smoking or wood-burning, tends to generate low molecular weight PAHs, whereas high-temperature

*Corresponding Author: m.khoshm@gmail.com, m-khoshsokhan@qom-iau.ac.ir (M. Khoshsokhan-Mozaffar)

industrial processes typically generate PAHs with higher molecular weights [8]. Hydrocarbon accumulation in the rhizosphere can challenge vegetation because growth parameters such as protein, carbohydrate, and lipid composition are related to photosynthetic activity of plants. PAH metabolism generates reactive electrophilic metabolites, which are the actual carcinogenic compounds that cause DNA (Deoxy nucleotide adenosine) damage [9]. Furthermore, PAHs trigger the production of reactive oxygen species and cell death in animal [10-12] and plant [13-15] cells. Alkio et al. (2005) and Liu et al. (2009) in separate works treated *Arabidopsis* species with phenanthrene. They indicated that Morphological changes and oxidative stress symptoms appeared due to phenanthrene treatment. In another study, increasing of anthracene remediation by transgenic tobacco plants was shown. Furthermore, high expression of fungal GST (Glutathione Transferase) was indicated as an index of anthracene tolerance [14]. Also, various plant species such as *Carexgracilis* [16], *Popolusspp*. [17-19], *Spirodella punctate* [20], have been shown to tolerate and phytodegrade xenobiotic pollutants [21]. Generally, xenobiotic detoxification in plants involves transformation, conjugation (for example with glucose or glutathione) and sequestration in the vacuole or cell wall [22]. However, the plant genes responsible for PAH uptake, degradation, and conjugation are largely unknown. Mechanisms of the PAH toxicity to plants are poorly understood, and the phytotoxicity appears to vary depending on the particular PAH and plant species [23, 24].

The objective of this study was to investigate the anthracene (a persistent 3-ring polyaromatic hydrocarbon present in dyes, wood preservatives) effect on physiological, morphological and anatomical changes of *Medicago sativa* (as a forage and economic plant) in hydroponic system.

MATERIALS AND METHODS

Seed germination, length and weight of seedlings

Seeds of *Medicago sativa* L. with germination potential were surface sterilized with 0.5% sodium hypochlorite for 20 min, thoroughly rinsed with deionized water and then germinated in rolls of neutral PH "germtest" paper under laboratory condition in Islamic Azad University of Qom. Anthracene was dissolved in acetone to prepare 1 mol L^{-1} anthracene stock. This stock was used for treatments. The seeds divided to four groups with four replicates: control, solvent (acetone), 2 mmol L^{-1} anthracene and 4 mmol L^{-1} anthracene [14]. After seven days germination percent, length, dry and fresh weight of seedlings was measured.

Plant materials, growth and treatment conditions

After seven days, the seedlings that normally germinated, were selected and transferred to pots containing hydroponic culture and kept there for 24 days, in order to adapt to hydroponic conditions. Growth environment temperature was set at about 27±3. In order to prevent the penetration of light, the pots were covered with aluminum foil. It should be noted, plants do not come into contact with aluminum. Medium pots were replaced every five days and every day the amount consumed from the pots was added. The culture was aerated with an air pump. Hoagland modified nutrient solution (1/2) contains macro and micro elements are as follows (pH =6):(in mmol L^{-1}):$Ca(NO_3)$ $2.4H_2O$:2.5; KNO_3:2.5; $MgSO_4.7H_2O$:1; KH_2PO_4:0.5, (Values in ppm): Fe-EDTA:3; H_3BO_3:0.5; Mn ($MnCl_2$.):0.5; Zn($ZnCl_2$):0.05 Cu($CuCl_2.2H_2O$):0.02; Mo(Na_2MoO_4):0.02

After 24 days of adaptation, the plants were treated with anthracene at concentrations of 2 mmol L^{-1} and 4 mmol L^{-1} for 12 days. Each of the four groups had five repetitions (pots) and each pot contained ten plants. The appearance of plants and roots were observed during the

growth stages. After the twelfth day, treatment and control samples (5 repetitions each) were harvested. A batch of them were washed thoroughly with deionized water, frozen in liquid N_2 and kept at 80°C for further analytical experiments. The second batch were fixated in FAA solution for anatomical studies.

Anatomical Studies

Cross sections of root and shoot in four groups were made by hand using commercial blades. The sections were stained with doubled carmine- methylene blue. Cortical thickness, the number of vascular bundles, etc. were observed using Nikon optical microscope.

Biochemical Analysis

All biochemical analyzes were carried out on the shoot and root separately (except chlorophyll on aerial parts). 200 mg of fresh tissues were homogenized in 3 mL of 0.1 mol L^{-1} potassium phosphate buffers, pH 6.8. Then, the homogenate was centrifuged at 12,000 g for 20 min and the supernatant was used as source of crude enzyme. All steps to obtain enzyme preparation were carried out at 4ºC.

The activity of SOD (superoxide dismutase) was determined by adding 50 µL the crude enzyme preparation to a solution containing 13 mmol L^{-1}metionine, 75 µmol L^{-1} p-nitro blue tetrazolium chloride (NBT), 100 nmol L^{-1} EDTA and 2 µmol L^{-1} riboflavin in a 50 mmol L^{-1} sodium phosphate buffer, pH 7.8 [25]. The reaction took place in a chamber under illumination the blue formazane produced by NBT photoreduction was measured by the increase in absorbance at 560 nm. The blank mixture had the same composition but it was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

Activity of APX (Ascorbate peroxidase) was measured according to the method of Nakano and Asada [26]. In brief, samples were homogenized in 1 mL of 50 mmol L^{-1} Na-phosphate buffer (pH = 7.8) containing 5 mmol L^{-1} ascorbate, 5 mmol L^{-1} DTT (Dithiothreitol), 5 mmol L^{-1} EDTA (Ethylene diamide tetra acetic acid), 100 mmol L^{-1}NaCl and 2% (w/v) PVP. The homogenate was centrifuged as 15000g for 15 min at 4°C. The reaction was initiated by adding H_2O_2. The reaction rate was monitored by the decrease in absorbance at 290 nm. Protein contents were determined by the method of Bradford [27] using BSA (Bovine serum albumin) as standard.

PO (Peroxidase) activity was determined by adding 100 µL of the crude enzyme preparation, diluted in the proportion of 1:25 (v/v), to 4.9 mL of a solution containing 25 mmol L^{-1} potassium phosphate buffer, pH 6.8, 20 mmol L^{-1}pyrogallol and 20 mmol L^{-1} H_2O_2. After incubation of the solution at 250C for 1 min, the reaction was stopped by adding 0.5 mL of 5% (v/v) H2SO4 and the absorbance was read at 420 nm [28]. The enzyme activity was calculated using the molar extinction coefficient of 2.47 mmol $L^{-1}cm^{-1}$.

The activity of PPO (Polyphenol oxidase) was determined using the same methodology described before for PO but omitting H_2O_2 from the reaction mixture. The enzyme activity was calculated as described before for PO.

GSTs activity was assayed spectrophotometrically at 25°C with reduced glutathione (GSH) and 1- chloro-2, 4-dinitrobenzene (CDNB) as substrates. This was done by watching an increase in absorbance at 340nm [29]. Malondialdehyde concentration was measured by the method of De Vos et al. (1991). MDA forms color complexes by combining with thiobarbituric acid (TBA)—its concentration being measured by a spectrophotometer [30].

The content of Chlorophylls a and b was estimated by the method described by Horwitz et.al (1990). Chlorophyll extraction from leaf material was carried out with 80% (v/v) acetone. The absorbance of resulting supernatant was recorded at 664 and 647 nm. The concentra-

tions of Chls a and b were calculated, using the following formulae [31]:

$$Chl\ a = [12.7\ (D\ 663) - 2.69\ (D\ 645)] \times V/1000 \times W$$

$$Chl\ b = [22.9\ (D\ 645) - 4.28\ (D\ 663)] \times V/1000 \times W$$

STATISTICAL ANALYSIS

Experiments followed a randomized complete block design. Three explants per pot and three replications per treatment were tested. Analysis of variance was performed by the General Linear Model procedure (SPSS ver. 16) and differences among treatments were evaluated by Duncan Test ($p \leq 0.05$).

RESULTS

Effect of anthracene on Seeds

The results showed that anthracene treatment didn't change germination percent, but seedling length and biomass (in 4 mmol L^{-1}) decreased but dry and fresh weight increased in 2 mmol L^{-1} after seven days of treatment (Figures 1 & 2).

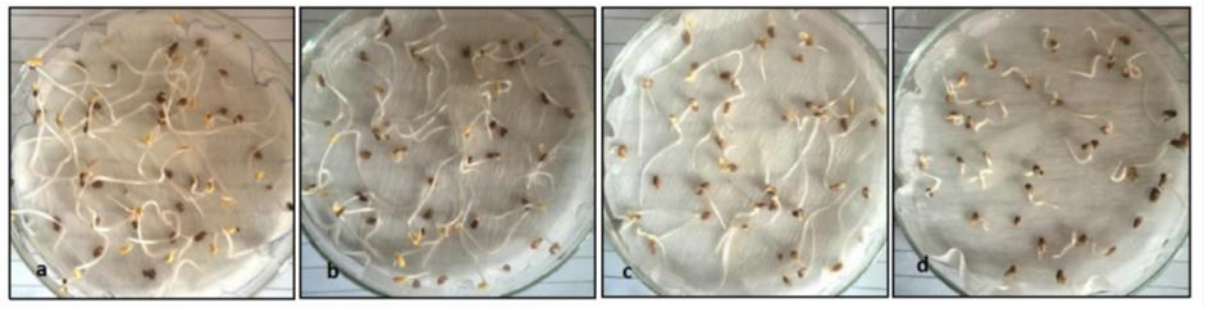

Figure 1. The effect of Anthracene treatment on germination and seedling length in (a) control (b) acetone (c) 2 mmol L^{-1} anthracene (d) 4 mmol L^{-1} anthracene after 7 days. (n=4)

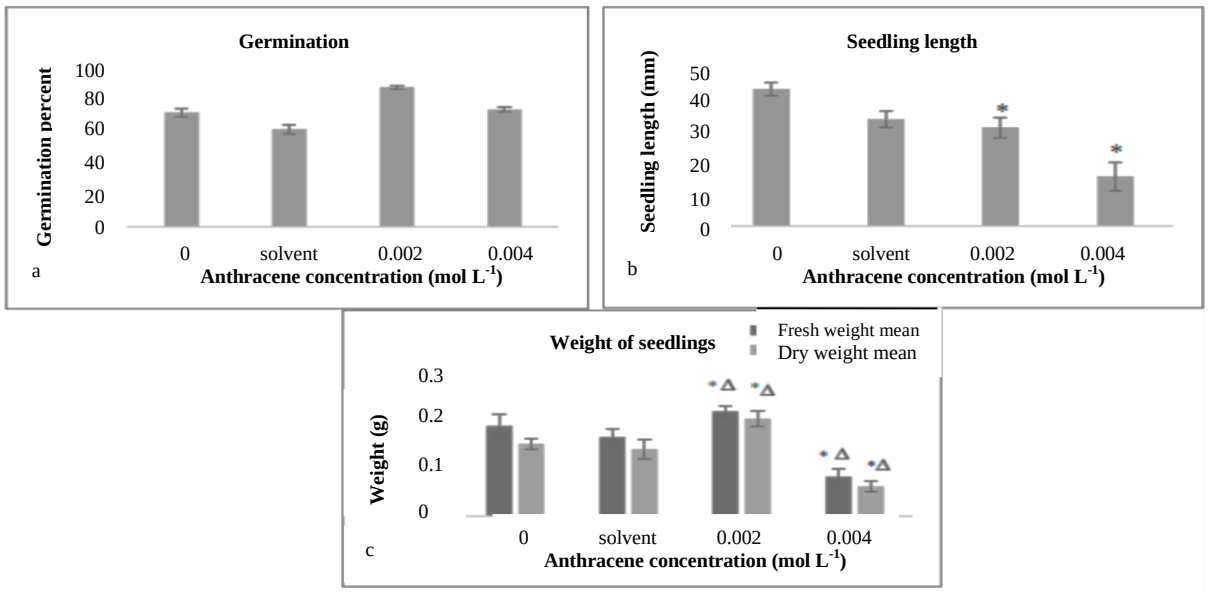

Figure 2. (a) Germination percent, (b) Seedling length and (c) Fresh and dry seedling weights of *Medicago sativa* under anthracene treatment. (n=4). error bars represent ± SD. Symbols * and Δ represent significant differences at $p \leq 0.05$ compared to the control and solvent (=acetone) groups, respectively.

Plant growth responses against anthracene

Plants which were treated with anthracene showed great stress features, e.g. the vitality and growth of the aerial shoots fell due to anthracene stress (Figure 3). Anthracene engulfed the roots so that they lost their steady state (Figure 4). The symptoms of stress increased, obviously, in 4 mmol L^{-1} anthracene. Also, the roots treated with anthracene increased number of hairs (Figure 5). Plants grown at a concentration of 2 mmol L^{-1} anthracene, were able to cope with stress. At 4 mmol L^{-1} anthracene, alfalfa seemed to have survived for more than two weeks.

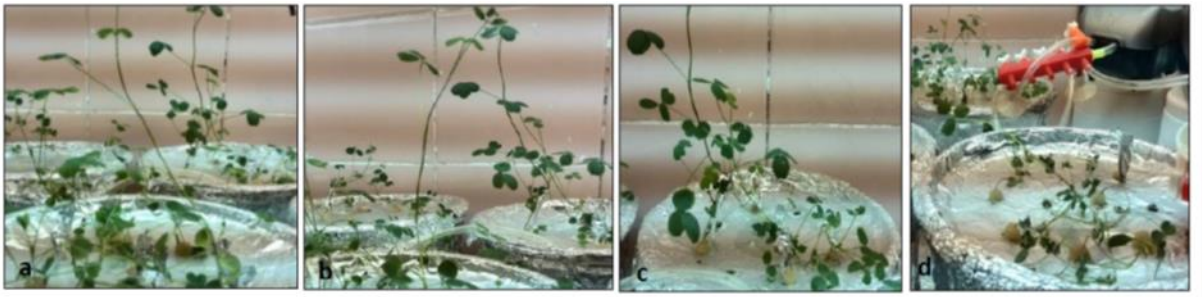

Figure 3. Aerial parts of plants in the 8th day of treatment. (a) control (b) Acetone (c) 2 mmol L^{-1} anthracene (d) 4 mmol L^{-1} anthracene.

Figure 4. Roots of plants on the 8th day of treatment: (left) control (right) treated with 4 mmol L^{-1} anthracene. The arrows show the coverage of anthracene around the roots.

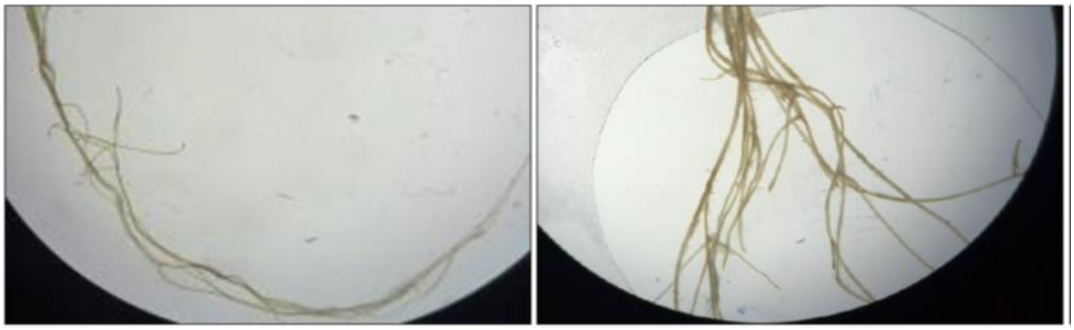

Figure 5. The root on the 8th day: (left) control and (right) treated with anthracene with 2 and 4 mmol L^{-1}.

Effect of Anthracene on Anatomical changes

Anthracene increased the thickness of periderm around the roots, reacting to stress with a cork-like covering, while the cross sections of the control group did not display the suberin layer around the root epidermis (Figure 6). The length of bundles in stem started to decrease after anthracene treatment (Figure 7).

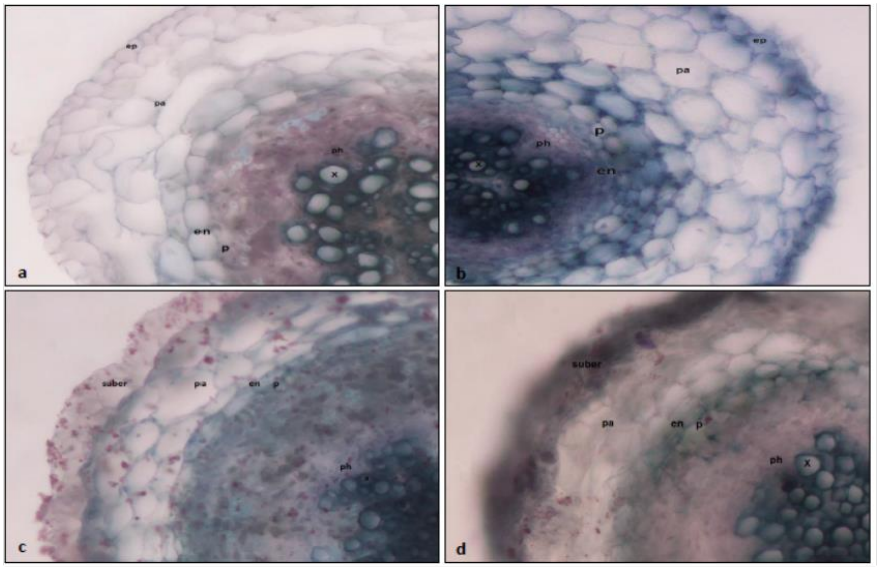

Figure 6. A cross-section of *Medicago sativa* roots (10X). a: control b: solvent (=acetone) c: 2 mmol L^{-1}anthracene treatment d: 4 mmol L^{-1} anthracene treatment. ep: the epidermis, pa: parenchyma, en: endoderm, p: pericycle, ph: phloem, x: xylem, suber: suberin.

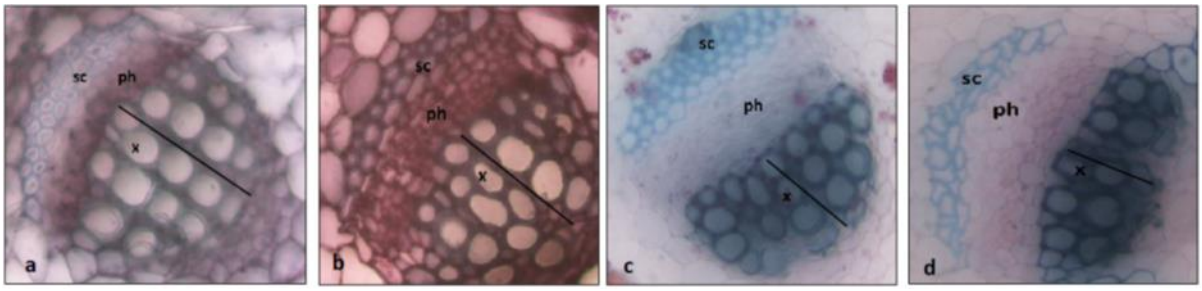

Figure 7. A view of the largest bundle in *Medicago sativa* leaf (40X) after 12 days. a: control b: solvent (=acetone) c: 2 mmol L^{-1} anthracene treatment d: 4 mmol L^{-1} anthracene treatment. ph: phloem, x: xylem, sc: sclerenchyma. The lines show the extent of xylem cells.

Effect of Anthracene on antioxidant responses

SOD: The results of measuring the activity of the superoxide dismutase in aerial shoots of *Medicago sativa* revealed that anthracene treatment led to an increase in the regular activity of this enzyme, significantly. This increasing at 4 mmol L^{-1} of anthracene was more obvious in shoot. Also, SOD activity elevation was observed in the roots of the treatment groups (Figure 8a).

SPO: anthracene treatment led to an increase in the regular activity of this enzyme in aerial organs. This elevation in the stems at 4 mmol L^{-1} was obvious. According to the results, activity changing of soluble peroxidase in roots in the treated groups was not significant at the $0.05 \geq p$. (Figure 8b).

PPO: The results of the enzyme in the stem indicated

that compared to control plants, the enzyme activity at 2 and 4 mmol L^{-1} anthracene is significantly increased (Figure 8c).

APX: According to the results of activity of the enzyme APX in shoots of alfalfa in 4 mmol L^{-1} anthracene compared to the control treatment, has increased as well. in roots treated in both 2 and 4 mmol L^{-1} anthracene the activity of this enzyme increased significantly (Figure 8d).

GST: The results of measuring of GST in shoots of alfalfa showed that treatment of anthracene on the glutathione transferase had no effect, significantly, treatment increased the enzyme activity in the roots (Figure 8e).

MDA: In shoots of alfalfa, treated with 2 and 4 mmol L^{-1} anthracene decreased MDA significantly and in contrast, the results of the measurements MDA in roots showed that treatment of anthracene increases MDA, compared to control plants (Figure 8f).

Chlorophyll content

Changes of chlorophyll a and b content in treated plants were not significant compared with control groups (Figure 8g).

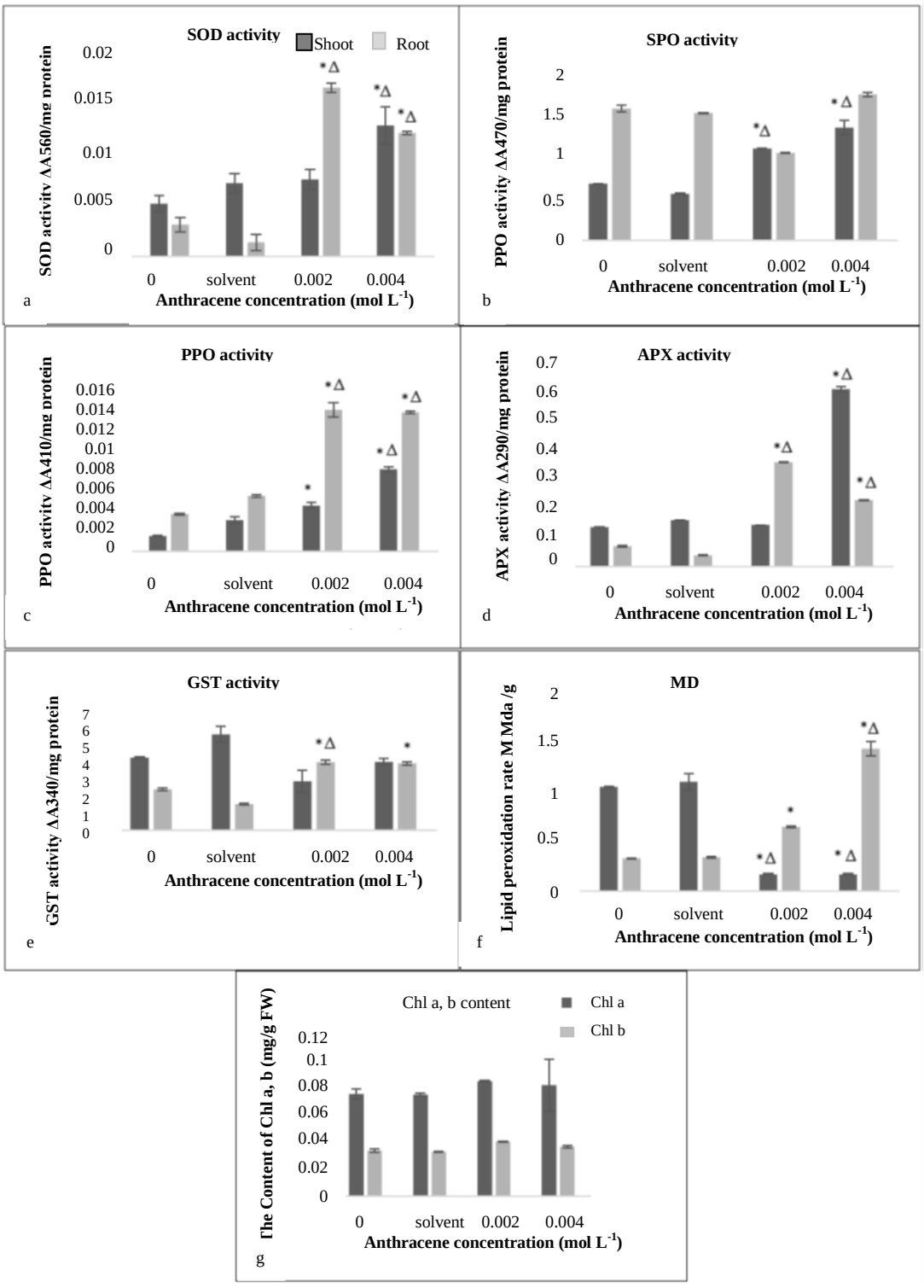

Figure 8. The effects of two concentrations of Anthracene on (a) SOD (b) SPO (c) PPO (d) APX (e) GST (f) MDA (g) Chl a, b. in shoot and root system after 12 days. Error bars represent ± SD. (n=5). Symbols * and Δ represent significant differences at $p \leq 0.05$ compared to the control and solvent (=acetone) groups, respectively.

DISCUSSION

Past studies reported phytotoxicity of PAHs to various plant species. Alkio et al. [13] documented several effects of phenanthrene to *Arabidopsis thaliana* including inhibition of growth and root development and induction of leaf lesions. In another study, Zhung et al. [32] showed Polynuclear aromatic hydrocarbons differentially influence growth of various on emergent wetland species. In other words, the different result is because of the different sensitivity of the plants species. In most studies, PAHs have no significant effect on germination [33, 34]. Paskova et al. [33], For instance, with the effect of PAHs and N-heterocyclic derivatives (NPAHs) on plants, showed only NPAHs have a negative effect on germination, but PAHs do not affect seed germination. In concordance with those, present investigation indicated anthracene treatment did not effect on seed germination (Figure 1) but subsequent growth of seedlings such as length, fresh and dry weight significantly decreased, especially in 4 mmol L^{-1} anthracene (Figure 2). According to this research, Smith et al. (2006) reported that germination of seven plant species from grasses and legumes in soil polluted with PHAs was unaffected but subsequent growth was significantly diminished[34]. Therefore, germination studies alone would not predict the success of subsequent growth of the species tested in the ranges of PHAs levels. Also, the biomass of seedlings at 2 mmol L^{-1} anthracene had significant increasing in compared with control plants (Figure 2c). It would appear that low concentration of anthracene simulates the growth of seedlings but more-concentration of anthracene inhibitsthe growth. Fan et al. (2008) reported that alfalfa root and shoot growth and dry weight were reduced in soil polluted with pyrene [35]. Also, Ahmadi et al. (2013) proposed that the biomass of three plant species was decreased with increasing the pollution level [36].

Alkio et al. (2005) reported that the transcript levels of expansin protein in phenanthrene treated *Arabidopsis* plants were reduced [13]. Expansins have known in cell wall loosening and cell enlargement. The reduction in expansin suggests thagrowth reduction may largely be due to inhibition of cellenlargement. Despite the importance of PAHs stresses in

Plants, limited information is available about the effect of PAHs stress on anatomical responses. In this study, anthracene was affected root and stem morphology and anatomy (Figures. 3-7). On the hydroponic cultivation of alfalfa with phenanthrene treatment, Flocco*et al.* (2002) reported that morphological symptoms such as leaf chlorosis and necrosis, lack of living plant cells and black roots with excessive branching can be observed [37]. It seems that plants try to increase their compatibility against stress, through developing lateral roots and root hairs to absorb water and minerals, so that their resistance is enhanced [38]. Furthermore, condensed anthracene around root hairs (especially in 4 mmol L^{-1}) acts as a barrier of water and nutrients. The severity of stress caused damage to the shoot at 4 mmol L^{-1} anthracene. Anatomical analysis indicated that anthracene treatment increased the thickness of periderm (a suberin layer) around root epiderm layer while such suberin layer was not observed in roots of control plants (Figure 6). It appears that presence of the periderm around the root was resulted more resistance against stress. Also, the diameter of xylem vessel bundles in stem of anthracene-exposed plants was decreased and this reduction in 4 mmol L^{-1} anthracene was higher (Figure 7). Zhan et al. (2015) suggested that higher concentration of phenanthrene in roots resulted the larger driving force for its transport from root to shoot [39], it appears that reduction of xylem vessel bundles diameter with increasing of anthracene concentration can be a defensive mechanism against the massive transfer of anthracene from root to shoot.

The effects of PAH Toxicants on oxidative stress in plants, are less documented. Our data, together with

some reports, indicated that PAHs exposure causes an oxidative stress response in plants [5, 15]. In this regards, plant and animal cells respond similarly to PHAs exposure, as shown by the production of H_2O_2 and localized cell death in *Arabidopsis*[13]. To prevent damage to cellular components by ROS, plant have developed a complex antioxidant system such as SOD, CAT (catalase), PO, APX and glutathione related enzymes [15]. Our results showed that anthracene treatment increased significantly the SOD, APX and PPO activities in root and shoot of *M. sativa* but just, increasing of PO in shoot system and glutathione transferase (GST) activities in root was significant (Figure 8). GST, a family of enzymes responsible for detoxification of a broad range of xenobiotics including herbicides by conjugating them with glutathione, may be a useful candidate for detoxification of PHAs[14]. It has been proposed that both PPO and PO play important roles in the metabolism of aromatic compounds in soil and water [37, 40]. Also, activity of antioxidant enzymes such as SOD, PO, CAT and APX increased with phenanthrene in *Arabidopsis* [15]. According to our results, it appears upregulation of antioxidant enzymes in shoot and root system of *M. sativa* can limit chemical damage due to high levels of ROS. Also, our data indicated that activities of root enzymes often increased significantly in both anthracene concentrations (2 and 4 mmol L^{-1}). Baldyga et al. (2005) reported that anthracene was present in all pea plant organs with its greatest amount in the roots. Therefore, high concentration of anthracene in root causes the beginning of an earlier oxidative stress response [41].

Anthracene treatment decreased significantly MDA level in shoot of *M. sativa* in both concentrations compared to the control plants while it increased significantly in root. High accumulation of anthracene and overcoming antioxidant system in roots causes increasing of lipid peroxidation and MDA level. Also, low MDA level in shoot can be achieved by anthracene accumulation in the alfalfa root and efficiency of photosynthetic system, as well as, modulated concentrations of antioxidant enzymes. Despite that Liu et al. (2009) reported that MDA level in leaf tissue of *Arabidopsis* after phenanthrene treatment increased significantly.

In the present study, no significant differences in total chlorophylls, chl a and chl b contents of alfalfa leaves of plant exposed to anthracene in compare to nonexposed ones observed. Our results agree with data reported by Flocco et al. (2002). He expressed that total chlorophyll contents of alfalfa leaves of plants exposed to 50 mg L^{-1}phenanthrene didn't change significantly at the end of experiment. Increased inhibition of photosynthesis during photomodification of anthracene was reported in the aquatic higher plant *Lemnagibba* [42].

According to obtain results, it appears that the photosynthetic apparatus of alfalfa can tolerate 2 mmol L^{-1} anthracene. However, in both concentrations (2 & 4 mmol L^{-1}), oxidative stress enzymes increased. Anthracene did not have a significant effect on germination, chlorophyll content, photosynthetic efficiency and plant vigor in aerial part at the 2mM anthracene.

CONCLUSIONS

The findings of this study support the some hypotheses. First, the sensibility of alfalfa, like some other members of Fabaceae family is not very high. Second, low water solubility of anthracene makes it less accessible to the plant roots and consequently, third, alfalfa can tolerate oxidative stress of 2 mmol L^{-1} anthracene and it can be as a monitoring plant for this PAH. This result could be applied for developing tolerance to recalcitrant environmental pollutants such as anthracene using transgenic approach and phytoremedation of PAHs.

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Conflict of intersts

The authors declare that there is no conflict of interests.

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