

High resistance of *Panicum miliaceum* L. to phenanthrene toxicity based on growth response and antioxidant system assessment

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Received December 04, 2020; accepted April 02, 2021.

Delo je prispelo 4. decembra 2020, sprejeto 2. aprila 2021.

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Abstract: Polycyclic aromatic hydrocarbons are a group of organic pollutants influencing different aspects of plants physiology. Physiological responses associated with the impact of phenanthrene (500, 1000, 1500, 2000 ppm) were analysed on *Panicum miliaceum* L. Seed germination was delayed in all treatments and 2000 ppm of phenanthrene (PHE) significantly retarded the germination rate (28 %) compared to control. The results revealed after 30 day of cultivation, only 1500 and 2000 ppm of PHE had negative impacts on growth parameters as well as photosynthetic pigment contents. Plants exposed to 500 and 1000 ppm of PHE showed an increase in the growth parameters without any symptoms of toxicity, indicating the high tolerance of seedlings to PHE. The activities of antioxidant enzymes were elevated in treated plants. In higher concentrations, H₂O₂ content also increased despite a reduction in malondialdehyde content. Furthermore, PHE had no effect on root phenol and shoot flavonoid contents and on shoot and root protein contents. Taken together, only higher concentrations of PHE triggered oxidative stress. It can be concluded PHE was not very toxic to *P. miliaceum* probably because of higher activity of antioxidant system involving in elimination of produced ROS even in plants treated by PHE higher concentrations.

Key words: polycyclic aromatic hydrocarbons; *Panicum miliaceum*; phenanthrene; physiological responses; toxicity

Velika odpornost navadnega prosa (*Panicum miliaceum* L.) na strupenost fenantrena temelji na rastnem odzivu in antioksidacijskem sistemu

Izvleček: Policiklični aromatski ogljikovodiki so skupina organskih onesnažil, ki vplivajo na različne vidike fiziologije rastlin. Fiziološki odzivi, povezani z vplivom fenantrena (PHE; 500, 1000, 1500, 2000 ppm) so bili analizirani na navadnem prosu (*Panicum miliaceum* L.). Kalitev semen je bila zapozneta pri vseh obravnavanjih, koncentracija fenantrena 2000 ppm je značilno zmanjšala hitrost kalitve (28 %) v primerjavi s kontrolo. Rezultati so pokazali, da se je negativen učinek fenantrena na rastne parametre in fotosinteza barvila v koncentracijah 1500 in 2000 ppm pokazal šele po 30 dnevih gojenja. Rastline, ki so bile izpostavljene koncentracijam 500 in 1000 ppm PHE so pokazale povečanje v rastnih parametrih, brez vsakršnih znakov zastrupitve, kar kaže na veliko tolerance teh rastlin na fenantren. V obravnavanih rastlinah se je povečala aktivnost antioksidacijskih encimov. Pri obravnavanjih z večjimi koncentracijami se je povečala tudi koncentracija H₂O₂, kljub zmanjšanju vsebnosti malondialdehida. Dodatno, PHE ni imel nobenega učinka na vsebnost fenolov v koreninah in flavonoidov v poganjkih, kot tudi ne na vsebnost beljakovin v koreninah in poganjkih. Zaključimo lahko, da fenantren ni bil zelo strupen za navadno proso, verjetno zaradi večje aktivnosti antioksidacijskega sistema, ki je preprečil tvorbo ROS, celo pri rastlinah obravnavanih z večjimi koncentracijami PHE

Ključne besede: policiklični aromatski ogljikovodiki; *Panicum miliaceum*; fenantren; fiziološki odziv; strupenost

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1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) belong to persistent organic pollutants (POPs) consisting of 2–7 condensed aromatic rings that are arranged in various structural configurations. They have been detected in various concentrations in different environments. These pollutants are derived from either natural or anthropogenic activities mainly associated with industrialization and urbanization (Krzebietke et al., 2018; Pogorzelec & Piekarska, 2018; Wiłkomirski et al., 2018). PAHs are mostly known for being highly toxic, mutagenic, teratogenic, and carcinogenic (Tian et al., 2019). PAHs hydrophobic nature leads to their accumulation and enrichment in soils, which is the reason for the necessity of the remediation of contaminated sites (Pretorius et al., 2018). Their physicochemical characteristics are low solubility in water, low vapour pressure, and highly lipophilicity which results in their high mobility in environment (IARC, 1983). The USEPA has categorized 16 mainly studied PAHs as priority pollutants with occurrence in environment (Mojiri et al., 2019). Phenanthrene (PHE), as one of the priority compounds presented in US-EPA list, represents a typical low molecular weight (LMW) PAH with three fused benzene rings that exists at high levels in PAH polluted environments (Fu et al., 2018). Increased environmental pollution and anthropological disturbances to ecosystems led to the study of abiotic stress responses in plants and subsequent concern about remediation of PAHs and ecosystem restoration strategies (Alkio et al., 2005).

Accumulation capacity and toxicity of PAHs in biota have resulted in a concern about their fate and transport in plants (Hamdi et al., 2007). Plants, as a dominant component of ecosystems, generally are first organisms exposed to the PAHs. They can uptake PAHs through the roots from contaminated soils and subsequently transfer them into shoots (Oguntimehin et al., 2010; Kummerová et al., 2013). There have been plenty of studies concerning the toxicity of PAHs on plants. It appears that all stages of plant growth and development can be influenced by PAHs, such as morphological, cytological, genetical, and metabolic disorders (Tomar & Jajoo, 2014; Dupuy et al., 2016). Inhibition of seed germination, reduction in biomass production, induction of oxidative stress, and disruption in photosynthetic apparatus function are some proven effects of PAHs on plants (Li et al., 2008; Liu et al., 2009; Tomar & Jajoo, 2014). A study carried out by Ahammed et al. (2012) has suggested that growth inhibition and biomass reduction by PAHs can be due to induction of oxidative stress. The oxidative stress induced by PAHs and its connection with morphological

disorders has been previously asserted by other studies, such as a study accomplished via *Arabidopsis thaliana* (L.) Heynh. exposed to PHE (Alkio et al., 2005). Plant morphology has been regarded as important indicators for toxicity measurements in some PAHs researches (Sverdrup et al., 2003; Kummerová & Kmentová, 2004; Tomar & Jajoo, 2014). However, biochemical changes can precede the growth and morphological changes. A better understanding of PAHs' influence on plant metabolic processes would provide data that is required for phytotoxicity assessment of these contaminants as well as selection of potential plants for the PAH phytoremediation in contaminated soils.

Panicum miliaceum L., also known as proso millet, broomcorn millet, etc., is an annual crop with thermophilic and photophilic characteristics which can adapt to various types of soils. Due to the resistance of *P. miliaceum* to salt, drought, and alkali stress, as well as its short growth cycle, it is usually grown as a remediation crop in various adverse conditions (Habiyaemye et al., 2017; Na et al., 2019). The previous studies on *P. miliaceum* have primarily described its tolerance/resistance to biotic and abiotic stresses (Hu et al., 2009). It has been reported that *P. miliaceum* is more tolerant to salt stress than foxtail millet, wheat, maize, or rice (Dong & Zheng, 2006; Liu et al., 2015). However, no report is available on the potential of *P. miliaceum* L. for evaluating its tolerance to PAHs and also its growth and physiological responses to PAHs' contamination. Considering the importance of this species as feed for livestock and birds and the subsequent entry of these compounds into the food chain, as well as a plant that is used directly in the human diet in some parts of the world (Sabir et al., 2011), this study was conducted to investigate the phytotoxicity of high concentrations of PHE on *P. miliaceum*. In other words, the evaluation of the biochemical and physiological responses of *P. miliaceum* plant and its resistance capability to PHE were the main purposes of this study.

2 MATERIAL AND METHODS

2.1 PLANT CULTIVATION AND TREATMENT

Experiment was carried out as a pot culture of plants using a completely randomized design with three replications for each treatment. Particular amounts of PHE were dissolved in ethanol and appropriate volume of PHE solution was added to perlite to give a final concentration of PHE (500, 1000, 1500 and, 2000 ppm). After thoroughly mixing, perlites were dried at room temperature (22–25 °C) to allow the ethanol evaporation for 72 h. Treated perlites were subdivided into 100 g portions in

plastic pots for plant cultivation. The aforesaid concentrations were selected due to the ineffectiveness of lower concentrations of PHE to plant based on initial screening experiments (Data not shown).

The seeds of *P. miliaceum* 'Pishahang' were purchased from Pakan Bazr (Isfahan, Iran) and stored at 4 °C until cultivation. Uniform and healthy seeds were selected and sterilized by soaking in 1 % (v/v) sodium-hypochlorite solution for 5 minutes, and rinsed thoroughly using sterile distilled water. Then, 20 disinfected seeds per pot (replicate) were planted in uncontaminated (control) and PHE-contained perlites. Then, they were germinated on watered perlites in a dark condition for three days at room temperature. Finally, pots with all germinated seeds were placed in growth chambers with controlled conditions (25-30 °C, 16/8 h light/dark photoperiod, light intensity of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by common day light fluorescent lamps, and relative humidity of 60 %) for four weeks. The water content of the pots was adjusted to 100 % field capacity every two days using sterile distilled water. After 4 and 10 days, the water of pots was replaced with 50 % and 100 % Hoagland solution, respectively. Seed germination rate was recorded. The rate of seed germination was defined as the percentage of germinated seeds to the total number of seeds.

2.2 HARVESTING OF PLANTS AND ASSAYS

The experiment was conducted for 30 days, and then plants were harvested and divided into the roots and shoots. Some morphological and growth parameters (such as germination, dry and fresh mass, root and shoot length) were estimated in order to evaluation of eventual differences between treatments. Plant samples were completely washed with water, immediately dried on the towel paper, and transferred to 70 °C after determining of the fresh mass (FM). The dry mass (DM) of samples was measured after 72 h. Three replicates of each root and shoot samples of different treatments were utilized for examination of biochemical and physiological parameters.

2.3 MEASUREMENT OF PHOTOSYNTHETIC PIGMENTS CONTENT

The assessment of the photosynthetic pigment contents (chlorophyll a, b, total chlorophyll, and total carotenoids) was performed based on the method described by Hartmut (1987). In brief, a quantity of 0.1g of fresh leaf samples was homogenized with 5 ml of acetone using a mortar and pestle on ice bath. After 48 hours, mixtures were filtered using a number 42 Whatman filter

paper and the absorbance of filtrates was recorded at 645, 663, and 470 nm by using a spectrophotometer (Analytic Jena, Specol 1500, Germany). The determined contents of chlorophyll a, b, total chlorophyll and carotenoids were expressed in $\mu\text{g g}^{-1}\text{M}$.

2.4 MEASUREMENT OF STRESS INDICATOR METABOLITES

2.4.1 Malondialdehyde

Malondialdehyde (MDA) content measured by a method described by Boominathan and Doran (2002). In order to preparation of extract, 0.1 g of plants fresh material was homogenate in trichloroacetic acid 0.1 % (TCA, Merck, Germany) and then centrifuged at 10000g for 5 min. Then, 0.5 ml of supernatants was mixed with 2 ml of 20 % TCA containing 0.5 % of 2-thiobarbituric acid (TBA, Merck, Germany) and transferred to hot water for 30 min at 95 °C. Mixtures cooled immediately and centrifuged at 10000g for 15 min. Finally, the absorbance of supernatants was recorded at 532 nm and MDA concentration were calculated according to a standard curve prepared using 3,1,1,3-tetraethoxy propane (0-100 nM). MDA content expressed as $\mu\text{mol g}^{-1}\text{FM}$.

2.4.2 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) assay was carried out according to Harinasut et al. (2003). Briefly, 0.1 g of plant fresh tissue was homogenized in TCA 0.1 % and centrifuged at 10000 g for 15 min. 0.5 ml of supernatant was immediately mixed with 0.5 ml of phosphate buffer (10 mM, pH = 7) and 1 ml potassium iodide (KI, 1 mM) and left at ambient temperature for 15 min. Then, the absorbance of extracts was recorded at 390 nm. The content of H_2O_2 was calculated using a standard curve prepared by using different concentrations of H_2O_2 (0-120 μmol) and expressed as $\mu\text{mol g}^{-1}\text{FM}$.

2.5 TOTAL PROTEIN CONTENT AND ACTIVITY OF ANTIOXIDANT ENZYMES

In order to preparation of extracts, 0.1 g of the plant fresh material was homogenized in phosphate-buffered solution (PBS, 50 mM, pH = 7) using mortar and pestle on ice bath and then centrifuged at 10000 g for 10 min at 4 °C. The supernatant was immediately used for quantification of the total soluble protein content using method described by Bradford (1976) as well as the activity of peroxidase (POD), Ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT). Each measurement was performed with three replications for each treatment.

The activity of POD (EC 1.11.1.7) was assayed by the method of Obinger et al. (1997). One unit of POD activity was defined as the enzyme amount capable of oxidizing of 1 μM guaiacol to tetraguaiacol per minute and the enzyme activity was expressed as U mg^{-1} protein.

The activity of APX (EC 1.11.1.11) was assayed according to of Boominathan and Doran (2002). One unit of enzyme activity is considered as the amount of enzyme required for production of 1 $\mu\text{l min}^{-1}$ of ascorbate. APX activity expressed as U mg^{-1} protein.

SOD (EC 1.15.1.1) activity was acquired by determination of nitro-blue-tetrazolium (NBT) photoreduction inhibition rate by extracts (Winterbourn et al., 1976). One unit of enzyme activity is defined as the amount of enzyme that inhibits 50 % of photochemical reduction of NBT. SOD activity was expressed as U mg^{-1} protein.

CAT (E.C. 1.11.1.6) assay conducted by using the method described by Chance & Maehly (1955). One unit of enzyme activity was considered as the amount of enzyme required for the decomposition of 1 μM H_2O_2 per min. The enzyme activity was expressed as U mg^{-1} protein.

2.6 QUANTIFICATION OF NONENZYMATIC ANTIOXIDANTS (TOTAL PHENOL, FLAVONOIDS AND ANTHOCYANIN CONTENTS)

For measurement of total phenolic and flavonoid contents, 0.1 g of plant samples were homogenized in 5 ml methanol using mortar and pestle. Then, the homogenates were centrifuged at 10000 g for 5 min and supernatants used for the tests. Assessment of flavonoids content has been accomplished based on a reported method by Chang et al. (2002). Briefly, 1.5 ml of methanol, 100 μl of 10 % aluminum chloride, 100 μl of 1 M potassium acetate, and 2.8 ml of distilled water were added to 500 μl of each extract (supernatant). After 40 minutes, the absorbance was recorded at 415 nm compared to the control. Quercetin was used for the preparation of calibration curve ($20\text{-}200 \text{ mg l}^{-1}$). The total flavonoid content of the extract was reported as milligram quercetin equivalents (QE) g^{-1} FM. The phenolic content has been evaluated by the method of Meda et al. (2005). 100 μl of the methanolic extract and 100 μl of Folin-Ciocalteu's reagent have been added to 2.8 ml distilled water, mixed thoroughly, and maintained for 6 min. Then, 2 ml of 20 % (w/v) sodium carbonate (NaHCO_3) was added and left at ambient temperature for 30 minutes in the dark. Finally, the absorbance was recorded at 720 nm by a spectrophotometer. The standard curve was prepared by using of different concentrations of gallic acid with the same procedure. The content of phenolics was calculated (mg ml^{-1}) by the measured absorbance based on calibration

line equivalent to the gallic acid (mg of GA g^{-1} of FM) (Meda et al., 2005).

To measure the total anthocyanin content, 0.05 g of dried plant sample was pulverized with 5 ml of hydrochloric acid containing 1 % methanol in a porcelain mortar. The solution was kept in the refrigerator for 24 hours and then, centrifuged for 10 minutes at 10000 g. The absorbance of supernatant was measured at 530 and 657 nm against the control (hydrochloric acid containing 1 % methanol). Finally, the anthocyanin content was calculated using the following equation (Mita et al., 1997).

$$A = A_{530} - (0.25 \times A_{657})$$

Where, A is absorbance of the solution (subscripts indicate the wavelength at which the absorbance is measured).

2.7 DPPH RADICAL SCAVENGING ACTIVITY

The method of Miliauskas et al. (2004) was used for DPPH radical scavenging capacity of plant samples. In order to, 0.1 g of plant fresh sample was extracted by 5 ml of methanol and then, centrifuged at 10000 g for 5 min. The supernatant was used for the test. To conduct the assay, 100 μl of extract was reached to 2 ml volume using methanol and 2 ml 0.0004 DPPH methanolic solution was added. The mixture was immediately mixed and incubated for 30 minutes in the dark at ambient temperature. The absorbance of the solution was recorded at 517 nm against control (2 ml DPPH and 2 ml methanol) and radical scavenging activity was calculated using the following equation:

$$I \% = (A \text{ control} - A \text{ sample}) / A \text{ control} \times 100$$

Where, A is absorbance of the solution.

2.8 DATA ANALYSIS

The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software (version 22) and expressed as mean \pm SD of three independent replications. Tukey's multiple range tests were applied for mean comparison and values of $p \leq 0.05$ were considered statistically significant. SPSS software was also applied to calculate the correlation coefficient (Pearson) between characteristics. Microsoft excel 2013 software was used for the preparation of figures.

3 RESULTS AND DISCUSSION

3.1 GERMINATION RATE

Seed germination was delayed by all PHE treatments for one day and the germination rate was decreased significantly as the concentration of PHE raised to 2000 ppm in comparison with the control ($p < 0.05$). Lower concentrations (500, 1000 ppm) of PHE had no effect on germination rate (Figure 1). Generally, seed germination is one of the most sensitive processes in the plants, using as a bioindicator for evaluation of pollutants effects on plants. Prevention of seed germination is one of the obvious toxic influence of PAHs (Henner et al., 1999; Somtrakoon & Chouychai, 2013) which has been frequently reported in plants such as soybean (Li et al., 2013), maize (Houshani et al., 2019), wheat (Wei et al., 2014), sunflower and alfalfa (Salehi-Lisar & Deljoo, 2015). Probably, the deterioration of seed embryo by PAHs is a main reason for the inhibition of seed germination (Reynoso-Cuevas et al., 2008; Kummerová et al., 2012).

3.2 GROWTH PARAMETERS

Plants exposed to PHE were morphologically more erect and up-righted at all levels in comparison with the control plants which were almost lying. The effect of PHE on growth parameters were depended on the type of parameter, plant organs as well as PHE concentration. After 30 days, root length only decreased at the highest concentration (2000 ppm) of PHE. Shoot length showed a significant increase in plants treated by 500, 1000 and 1500 ppm of PHE ($p < 0.05$), but did not show a significant change at 2000 ppm in comparison with the con-

rol (Figure 2a). Shoot FM increased significantly at 1000 and 1500 ppm, but showed a significant decrease at 2000 ppm of PHE ($p < 0.05$). However, it had no notable increment at 500 ppm (lowest level). Both 500 and 1000 ppm of PHE led to considerable increase in root FM, but 1500 and 2000 ppm concentrations caused no significant enhancement ($p < 0.05$) (Figure 2b). Root and shoot DM were not remarkably affected by PHE, except for shoot DM at 500 ppm of PHE which increased significantly ($p < 0.05$) (Figure 2c).

Our results showed that the highest treatment of PHE significantly increased fresh and dry weight ratios of root to shoot (R/S) ($p < 0.05$) (Figure 2d).

Growth parameters such as plant height, biomass, and leaf area represent a function of plants' growth (Li et al., 2013). The reduction of the growth indices in the presence of PAHs has been demonstrated previously in plants such as rice (Li et al., 2008), wheat (Tomar & Jajoo, 2014; Salehi-Lisar & Deljoo, 2015), maize and pea seedlings (Kummerová et al., 2012). In the mentioned studies, the applied concentrations of PAHs were mostly below 100 mg kg⁻¹. Rice plants had good resistance to slightly higher concentrations of PAHs (100 and 200 mg kg⁻¹ of pyrene and PHE), while the addition of 400 mg kg⁻¹ of pyrene and PHE resulted in some negative impacts on this plant growth (Li et al., 2008). In our work, intriguingly, *P. miliaceum* plants not only grew without any symptoms of toxicity at 500 and 1000 ppm of PHE, but also showed an increase in the growth parameters; indicating the high tolerance of *P. miliaceum* seedlings to PHE. Although the higher concentrations of PHE (1500 and 2000 ppm) reduced the germination rate of *P. miliaceum* seeds, it is presumed that 1500 ppm is the highest concentration of PHE that *P. miliaceum* could tolerate

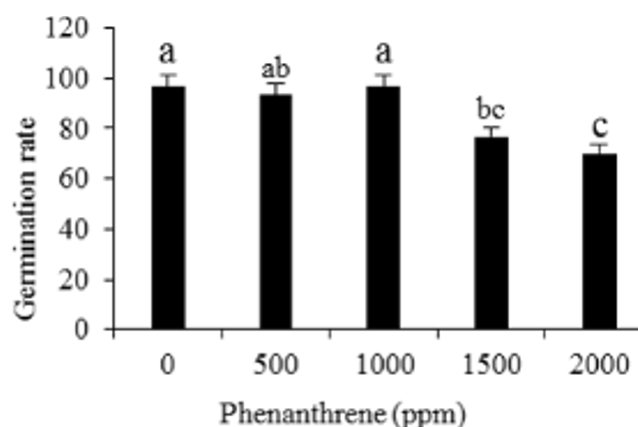


Figure 1: The effect of different concentrations of phenanthrene on seed germination rate of *P. miliaceum*. The level of confidence is 95% according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

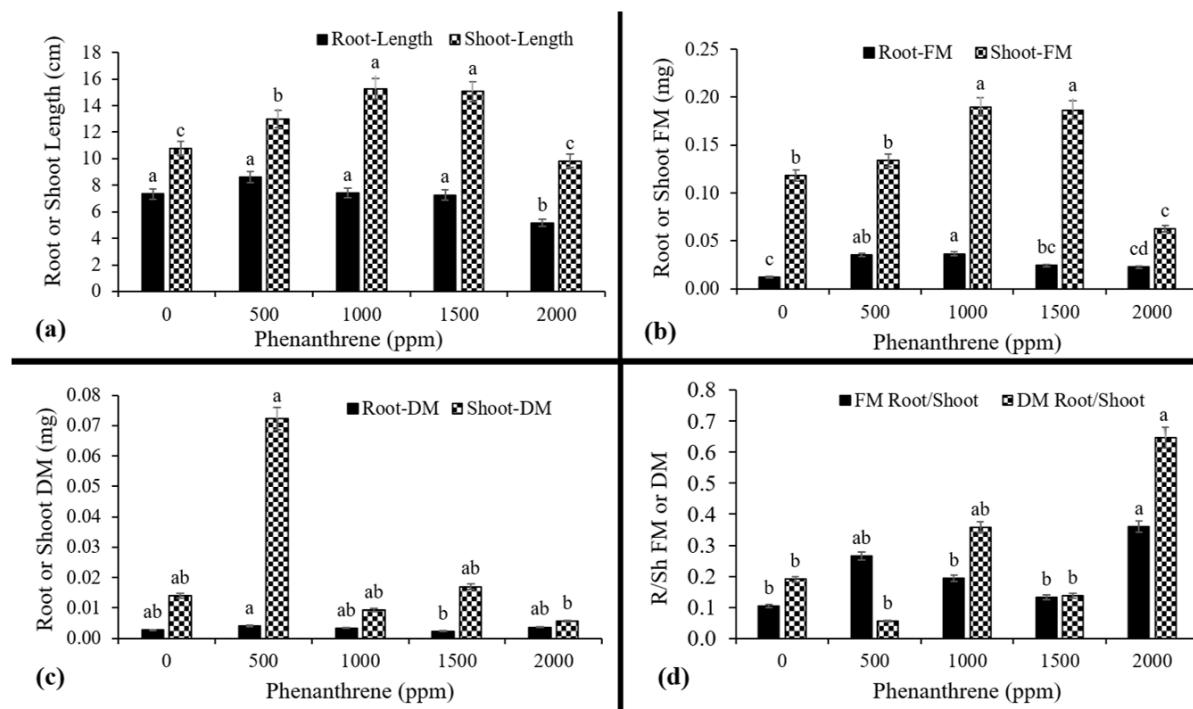


Figure 2: The effect of different concentrations of phenanthrene on *P. miliaceum* growth parameters a) root and shoot length, b) root and shoot fresh mass, c) root and shoot dry mass, and d) root to shoot fresh or dry mass ratios). The level of confidence is 95 % according to Tukey Test ($n = 3$ replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

without negative effects on growth. At this concentration the growth parameters are either increased or similar to control plants. However, at 2000 ppm of PHE, shoot fresh mass was decreased by 50 %. In spite of this, *P. miliaceum* was able to survive in presence of 2000 ppm of PHE during the testing period. The previous studies on *P. miliaceum* have also primarily described its resistance to various adverse conditions compared to similar plants from Poaceae family such as foxtail millet, wheat, maize, or rice (Dong & Zheng, 2006; Hu et al., 2009; Liu et al., 2015).

The data showed a noticeable increase in R/S ratio of *P. miliaceum* exposed to the highest concentration of PHE (2000 ppm). Literature review revealed that it is sometimes important for plants to allocate carbon resources to roots under stress condition such as PAHs toxicity. Plant biomass allocation, as a similar response induced by PAHs, was previously reported in *Zea mays* L. and *Phragmites australis* (Cav.) Trin. ex Steud. (Nie et al., 2010; Dupuy et al., 2016). The same results with increasing R/S ratio were acquired in *Medicago sativa* L., clover and maize impressed by PAHs (Desalme et al., 2011; Dupuy et al., 2015; Salehi-Lisar & Deljoo, 2015; Afegbua & Batty, 2018). Altogether, the high R/S ratio along with a significant decrease in root length in present work is a

reason for the increased root thickness. The increment in root thickness was also confirmed for *Pisum sativum* L. and *Z. mays* plants treated with fluoranthene (Kummerová et al., 2013). In maize, PHE induced the extensive suberization of endo- and exodermises as a protective response by the plant for reduction of PHE penetration into roots (Dupuy et al., 2016).

3.3 PHOTOSYNTHETIC PIGMENT CONTENTS

Chlorophyll a, chlorophyll b and total chlorophyll were remarkably reduced in the plants treated by PHE compared to the control ($p < 0.05$). The lowest content of chlorophyll a and b were observed in the plants exposed to 1500 and 2000 ppm of PHE, respectively (Figure 3 a & b). Moreover, the increase in chlorophyll a/b ratio at all levels (especially 2000 ppm) of PHE in comparison with the control was statistically significant ($p < 0.05$) (Figure 3c). Also, plants treated with 1500 ppm of PHE showed a notable decline in carotenoids content in comparison with the control ($p < 0.05$) (Figure 3d). It appears that chlorophyll b was more affected by PHE, but carotenoids were less sensitive compared to chlorophylls (Figure 3).

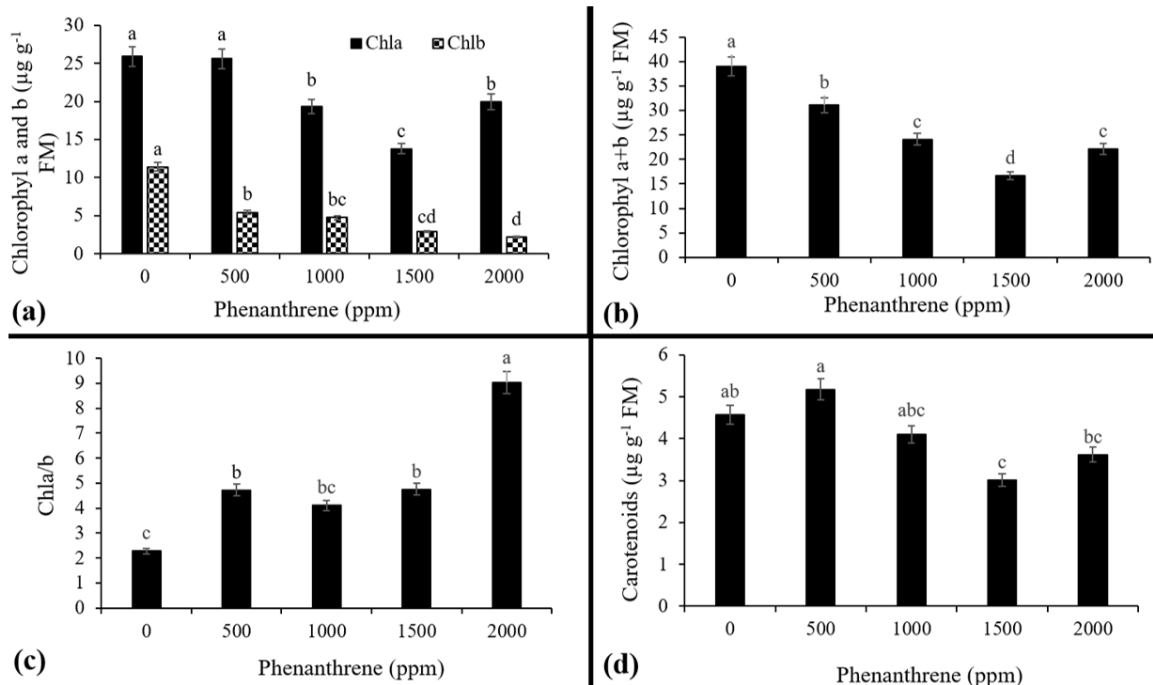


Figure 3: The effect of different concentrations of phenanthrene on photosynthetic pigments contents of *P. miliaceum* plants a) Chla and b, b) Chl a+b, c) Chla/b, and d) Carotenoids. The level of confidence is 95 % according to Tukey Test ($n = 3$ replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

According to the results, photosynthetic processes in *P. miliaceum* were probably inhibited by PAH. This was demonstrated by the decrease of Chl a, b and total chlorophyll, and by the increase in the Chl a/b ratio at all treatments. However, based on the data obtained by present work, growth parameters generally increased or at least maintained at control level, and only the highest concentration of PHE led to reduction in some traits. Biomass production formerly regarded as a reliable external indicator of internal status of plant photosynthesis processes (Tomar & Jajoo, 2014). Accordingly, it can be suggested that most likely changes in photosynthetic pigments content cannot be an appropriate indicator for assessing the sensitivity or resistance of *P. miliaceum* to PAHs contamination. This assumption was also supported by results of Salehi-Lisar & Deljoo (2015). Reduction of photosynthetic pigments content have been reported in *Arabidopsis thaliana* and wheat plants treated by PHE and fluorene, respectively (Liu et al., 2009; Tomar & Jajoo, 2014).

PAHs can cause oxidative stress in plants through induction of ROS production. In addition to structural role and light absorption, carotenoids can directly inactivate ROS or indirectly prevent the formation of ROS

by quenching chlorophyll-elicitation (via xanthophyll cycle) and protect chloroplast membranes against oxidative stress (Ramel et al., 2012). Therefore, it seems that preservation of carotenoid content, especially at lower concentrations of PHE, has also contributed to plant tolerance for this pollutant.

3.4 MALONDIALDEHYDE AND HYDROGEN PEROXIDE CONTENTS

A considerable dose-dependent elevation in H_2O_2 content was observed after 30 days of plants treatment by PHE. As compared to the control and the other PHE treatments, plant treated by 2000 ppm of PHE showed a significant increase in root H_2O_2 content ($p < 0.05$). 1500 and 2000 ppm of PHE also caused a notable enhancement in shoot H_2O_2 content in comparison with the control plants ($p < 0.05$) (Figure 4a). 1000 ppm of PHE led to a significant increase in root MDA content, compared with the control and other PHE treatments ($p < 0.05$). Also, a significant reduction in shoot MDA content was observed in plants treated by 1000, 1500 and 2000 ppm compared to the control plants ($p < 0.05$) (Figure 4b).

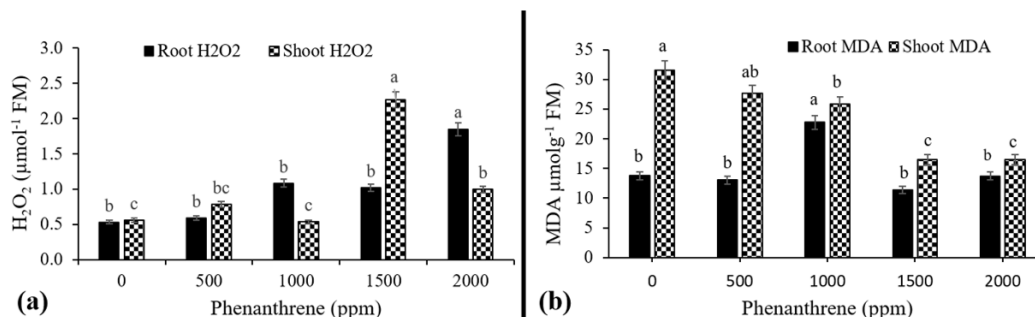


Figure 4: The effect of different concentrations of phenanthrene on H₂O₂ and MDA contents in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

In general, with increment in PHE concentrations, H₂O₂ content was raised in the shoots and roots of *P. miliaceum* compared to the control plants. The increased amount of H₂O₂ was previously reported in the seedlings of wheat (Wei et al., 2014) and *Arabidopsis thaliana* (Liu et al., 2009). The highest amount of this trait was obtained at 1500 and 2000 ppm of PHE for roots and shoots, respectively. However, the amount of MDA mounted to the highest level in 1000 ppm, and then dramatically dropped in higher concentrations of PHE in the roots ($p < 0.05$). In shoots, the amount of MDA was decreased compared to control. Taken together, given that even in non-stress conditions, there is always some H₂O₂ in plant tissues; here, it appears that the enhancement in H₂O₂ amount was not a big challenge for plant or its antioxidant system would be able to scavenge produced H₂O₂. So, not only the content of MDA did not increase, it even decreased. According to the results, some growth parameters even increased at 1500 treatment. It can be suggested that at 2000 ppm of PHE plant allocated more photosynthetic products to the root, for its thickening in order to create barriers against PHE penetration. Analysis of PHE concentration using HPLC also showed a decrease in the penetration of PHE into the root in plants treated by 2000 ppm of PHE (Data not shown). In addition, a 50 % reduction in shoot FM and increased root FM, along with a significant reduction in its length, also supports this point of view. So, it can be assumed that the antioxidant system of the root is strong enough and has been able to reduce the stress effects of PHE on plants. On the other hand, given that at high concentrations of PHE, the accumulation of MDA was lower in roots, it indicates that the potency of PHE stress in *P. miliaceum* is not considerable. Therefore, it can be concluded that MDA accumulation due to oxidative stress could not be a reliable marker for evaluation of the negative effect of PHE on *P. miliaceum*.

3.5 TOTAL PROTEIN CONTENT

Generally, PHE showed no significant effect on shoot and root protein content, however at the highest concentration of PHE a significant fall in shoot protein was observed ($p < 0.05$) (Figure 5). Therefore, the decrease in growth observed at this concentration of PHE may also be due in part to the plants' low capacity to produce protein under PHE toxicity. However, it is also possible that the transfer of more photosynthetic products to the root, and hence, the decrease in shoot growth, has led to a decrease in plants protein content. It is well known that plants need both carbohydrates and proteins to grow. In addition, root thickening may act as a barrier to efficient and effective uptake of nutrients and in turn lead to reduced growth. Salehi-Lisar & Deljoo (2015) has also reported a decrease in shoot and root soluble protein in sunflower plants treated by fluorene.

3.6 ANTIOXIDANT ENZYMES ACTIVITY

The activity of antioxidant enzymes were assessed in *P. miliaceum* 30 days after seed cultivation. A dramatic increment was observed in the SOD, POD, CAT, and APX activities in plants treated by 1500 and 2000 ppm of PHE ($p < 0.05$) in both shoots and roots ($p < 0.05$). However, there was often a significant difference between 1500 and 2000 treatments and generally the highest activity of these enzymes observed at 2000 ppm of PHE, except for CAT activity in roots which was the highest in 1500 ppm of PHE ($p < 0.05$) (Figure 6a-d). In general, antioxidant enzymes' activities in roots were higher than those in shoots of the plants (Figure 6a-d).

ROS accumulation is a main factor involving in toxicity induction due to direct or an indirect outcome of the PAHs exposure. ROS stimulates the activity of anti-

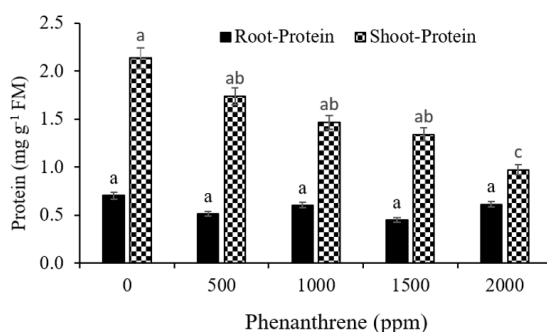


Figure 5: The effect of different concentrations of phenanthrene on protein content in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

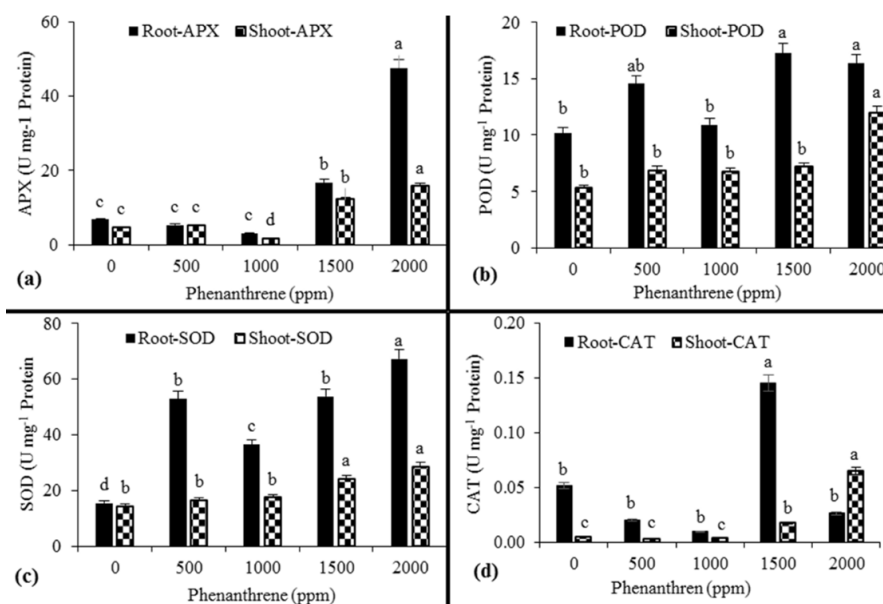


Figure 6: The effect of different concentrations of phenanthrene on antioxidant enzymes activity (U mg^{-1} protein) in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD. The same letters above the bars indicate no significant differences ($p < 0.05$).

oxidant enzymes (e.g. SOD, POD, CAT, and APX). These enzymes are responsible for scavenging of produced ROS and their activities may indicate stress resistance potential of plants (Di Giulio, 1991). Increased activity of antioxidant enzymes by PAHs toxicity has been previously reported in maize (Houshani et al., 2019), wheat, sunflower, and alfalfa (Salehi-Lisar & Deljoo, 2015), rice (Li et al., 2008) treated by fluorene, pyrene and PHE, respectively. In present study, higher activity of antioxidant enzymes in plants treated by 1500 and 2000 ppm of PHE is a result of induced ROS generation by PHE. However, PHE induced a concentration-dependent oxidative stress

and the activity of antioxidant enzymes did not changed significantly in the lower PHE treatments. The analysis of correlation showed negative correlation coefficient between MDA content and antioxidant enzyme activities in shoot (SOD $r^2 = -0.893$, POD $r^2 = -0.590$, CAT $r^2 = -0.705$, APX $r^2 = -0.740$) and root (SOD $r^2 = -0.771$, POD $r^2 = -0.717$, CAT $r^2 = -0.451$, APX $r^2 = -0.688$) (Table 1). These findings indicate that antioxidant enzymes involved in ROS detoxification and plants resistance to oxidative stress are induced by PHE toxicity. Similar results were obtained for maize, alfalfa, sunflower, and wheat plants (Salehi-Lisar & Deljoo, 2015).

3.7 NONENZYMATIC ANTIOXIDANTS

PHE significantly increased anthocyanin and total phenol contents in shoots of plants treated by 500 and 2000 ppm and 1500 ppm, respectively, compared to the control and other PHE treatments ($p < 0.05$). PHE had no effect on root anthocyanin and total phenol contents. However, flavonoids contents elevated at 2000 ppm of PHE only in roots ($p < 0.05$) and no remarkable changes were detected in shoots (Table 2). This increment can be interpreted as a defence mechanism. Plants requiring a protective system involving enzymatic and non-enzymatic mechanisms for detoxification of ROS (Alscher et al., 1997). In this work, the amount of non-enzymatic antioxidants in shoot or root was raised probably in order to detoxification of accumulated oxidant metabolites at

higher concentrations of PHE, along with increased antioxidant enzymes' activity, resulting in ROS scavenging and finally reduction in oxidative stress severity.

3.8 DPPH RADICAL SCAVENGING ACTIVITY

As seen in Figure 7, there were no significant changes in DPPH radical scavenging activity between treatments. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) a molecule containing a stable free radical widely used to study the free radical-scavenging activity of natural antioxidants (Brand-Williams et al., 1995). According to the results of this work it can be concluded that studied plant was able to maintain their antioxidant capacity as to control plants.

Table 1: Statistical analysis for correlation between the activity of antioxidant enzyme and MDA content in the shoot and root of *P. miliaceum* plant.

	APX Root	APX Shoot	SOD root	SOD Shoot	POD Root	POD Shoot	CAT Root	CAT Shoot	MDA Root	MDA Shoot
MDA Shoot	0.688**	0.740**	0.771**	0.893**	0.717**	0.590*	0.451 ^{ns}	0.705**	0.192 ^{ns}	1
MDA Root	0.304 ^{ns}	0.563*	0.299 ^{ns}	0.142 ^{ns}	0.532*	0.152 ^{ns}	0.490 ^{ns}	0.254 ^{ns}	1	
CAT Shoot	0.978**	0.875**	0.679**	0.801**	0.527*	0.784*	0.022 ^{ns}	1		
CAT Root	0.041 ^{ns}	0.196 ^{ns}	0.074 ^{ns}	0.238 ^{ns}	0.397 ^{ns}	0.130 ^{ns}	1			
POD Shoot	0.688**	0.733**	0.791**	0.747**	0.538*	1				
POD Root	0.554*	0.767**	0.816**	0.730**	1					
SOD Shoot	0.797**	0.767**	0.812**	1						
SOD Root	0.641*	0.772**	1							
APX Root	0.873**	1								
APX Shoot	1									

Notes: **Correlation is significant at 0.01 levels, *Correlation is significant at 0.05 levels, ns correlation is not significant.

Table 2: The effect of the different concentrations of phenanthrene on phenol (mg GAE g⁻¹ FM), flavonoids (mg QE g⁻¹ FM) and anthocyanin (mg QE g⁻¹ FM) contents in the shoot and root of *P. miliaceum* plant.

Phenanthrene (ppm)	Root			Shoot		
	Phenol	Flavonoids	Anthocyanin	Phenol	Flavonoids	Anthocyanin
0	2.93 ^a ±0.17	0.27 ^b ±0.00	0.03 ^a ±0.01	0.73 ^b ±0.32	1.10 ^a ±0.07	0.18 ^b ±0.17
500	3.03 ^a ±0.03	0.27 ^b ±0.00	0.05 ^a ±0.02	1.17 ^b ±0.99	1.30 ^a ±0.05	0.50 ^a ±0.17
1000	1.95 ^a ±0.58	0.28 ^b ±0.00	0.04 ^a ±0.01	1.22 ^b ±0.16	1.20 ^a ±0.04	0.16 ^b ±0.17
1500	2.07 ^a ±1.47	0.27 ^b ±0.00	0.05 ^a ±0.01	7.85 ^a ±0.26	1.04 ^a ±0.20	0.15 ^b ±0.17
2000	2.06 ^a ±0.29	0.30 ^a ±0.01	0.02 ^a ±0.00	1.03 ^b ±0.12	1.14 ^a ±0.06	0.39 ^a ±0.06

The data represent the mean of three replications ± SD and similar upper case letters indicates no significant difference at $p < 0.05$.

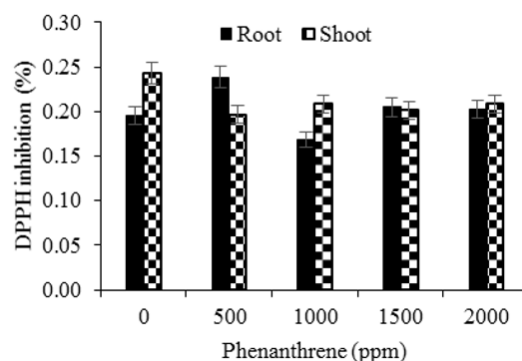


Figure 7: The effect of different concentrations of phenanthrene on DPPH radical scavenging activity in the shoot and root of *P. miliaceum* plant. The level of confidence is 95 % according to Tukey Test (n = 3 replicates) and error bars indicate SD.

4 CONCLUSIONS

The measurements of some morphological parameters as well as cellular responses in the forms of enzymatic and non-enzymatic antioxidants' activities in *Panicum miliaceum* exposed to phenanthrene have been main objectives of presented work. PHE had a negative impact on germination rate, growth and pigment content of *P. miliaceum* only at higher concentrations (1500 and 2000 ppm). Therefore, in comparison to other studied plants *P. miliaceum* showed relatively very high tolerance to PHE toxicity. The results also showed a noticeable increment in antioxidant system activity due to the toxicity caused by PHE at higher concentrations as well as a significant elevation in non-enzymatic antioxidant contents that can be explained as a probable contributory system to the enzymes in order to ROS detoxifying. Taken all together, the antioxidant system of *P. miliaceum* was probably strong enough to reduce toxicity of produced ROS in significant amount, thus plant survived even in higher concentrations of PHE. It would be worthwhile to examine the capability of this plant for phytoremediation purposes through further research.

ACKNOWLEDGMENT

The authors thank the Research Affairs of University of Tabriz for financial and other supports.

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