

Enzymatic Activity of Alfalfa in a Phenanthrene-contaminated Environment

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Abstract—This research was undertaken to study enzymatic activity in the shoots, roots, and rhizosphere of alfalfa (*Medicago sativa* L.) grown in quartz sand that was uncontaminated and contaminated with phenanthrene at concentrations of 10 and 100 mg kg⁻¹. The higher concentration of phenanthrene had a distinct phytotoxic effect on alfalfa, inhibiting seed germination energy, plant survival, and biomass accumulation. The plant stress response to the environmental pollution was an increase in peroxidase activity. Peroxidases were the predominant enzymes in the alfalfa shoots and roots. The peroxidase profile in the shoots differed from that in the roots and had different isoenzyme numbers. 2,2'-Azinobis-(3-ethylbenzo-thiazoline-6-sulphonate) (ABTS) peroxidase was predominant in the shoots, and 2,7-diaminofluorene (2,7-DAF) peroxidase was predominant in the roots. Under the influence of phenanthrene, the activity of 2,7-DAF peroxidase increased in the shoots, and the activity of ABTS peroxidase increased in the roots. Alfalfa root peroxidases were the prevalent enzyme systems in the rhizosphere sand. Examination of the activity of alfalfa root peroxidase toward phenanthrene revealed the possibility of involvement of the plant enzyme in rhizosphere degradation of the PAH.

Keywords—*Medicago sativa*, enzymatic activity, peroxidase, phenanthrene.

I. INTRODUCTION

OVER the past 10 to 15 years, phytoremediation, a relatively new environmental-cleanup biotechnology based on the use of plant-microbial associations, demonstrated its success and strength because of its being cheap, esthetically attractive and efficient. As a result of active scientific research and field-trial experience, it has been established that there are effective phytoremediating plants capable of intensive elimination of both organic and inorganic pollutants from contaminated soil. For example, alfalfa (*Medicago sativa* L.) is recognized as an effective phytoremediating species among plants promising for cleanup of soils polluted by polycyclic aromatic hydrocarbons (PAHs) [1]-[3]. Studies of the mechanisms responsible for alfalfa-based phytoremediation of hydrocarbon-contaminated soil have shown that this plant can selectively increase the number of PAH degraders in its rhizosphere [3], [4], thereby intensifying microbial degradation of pollutants in soil. A principal factor of enhanced degradation of hydrocarbons in the plant

rhizosphere during phytoremediation is the rhizosphere effect, i.e., increased numbers and activity of soil microorganisms in the plant-root zone. However, the contribution of plant-root extracellular enzymes to the rhizosphere degradation of organic pollutants seems considerable, with account taken of the findings of Gramss et al. [5], who showed that the roots of some plants release enough oxidoreductases to take part in the oxidative degradation of certain soil constituents. Active involvement of plant peroxidase in the phytoremediation process has been suggested by several authors [1], [6], [7]. With this in mind we hypothesize that the particular efficiency of alfalfa in phytoremediation of PAH-contaminated soil is connected not only with the selectively increased numbers of PAH degraders but also with the plant's own enzymatic activity toward aromatic contaminants.

The objectives of this study were to reveal of alterations in the oxidoreductase activity of alfalfa grown under the influence of the three-ring aromatic hydrocarbon phenanthrene.

II. MATERIALS AND METHODS

A. Plant cultivation

Seeds of alfalfa (*Medicago sativa* L.) were obtained from the Scientific Research Institute of Agriculture in the South-East (Saratov, Russia). Ten calibrated and surface-sterilized seeds were planted in each pot. There were three replicates of each treatment in our experiment.

Alfalfa plants were cultivated in uncontaminated (control) and phenanthrene-containing sand. Two phenanthrene concentrations were applied to heat-sterilized quartz sand (particle size 1–2 mm) by spraying 0.15 and 1.5% (w/v) acetic solutions to final phenanthrene concentrations of 10 and 100 mg kg⁻¹, respectively. The control substrate was treated with pure acetone of equal volume. After solvent evaporation, substrates were transferred to pots containing 1.5 kg of quartz sand. Each pot was equipped with a plastic bag of appropriate size. At harvest, the complete root system with attending substrate could be removed by lifting the bag out of the pot.

Plants were cultivated in a growth chamber with a 14/10 hours day/night regimen (light intensity: 8000 lux, temperature: 24/20 °C, relative humidity: 70%) for 4 weeks. The water content of the quartz sand was maintained at 80% of the maximum water-holding capacity by adding deionized water or Ruakura nutrient solution, according to [8].

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B. Plant Analyses

After 8 weeks of cultivation, the plants were lifted out of the pots, the plastic bags were removed, and the plants were transferred into a 2-l beaker. The plant roots were dipped gently, and the shoots were separated from the roots and were dried at 70°C until constant dry weight.

The phytotoxicity of the phenanthrene-contaminated sand was assessed by the seed germination rate, germination energy, and growth and development of the roots and shoots. The germination rate is a measure of the speed of germination. In this study, it was estimated as the percentage of seeds that had germinated over one-half of the period used for estimation of the germination rate.

C. Preparation of Extracts

Shoot and root samples (0.2 g) were ground in a mortar with quartz sand and were suspended in 5 ml of 0.2 M Na/K-phosphate buffer (pH 6.0). The homogenate was centrifuged (5000 g for 10 min), and the supernatant liquid was filtered (0.2 µm) and dialyzed for 24 h against 2 l of distilled water.

D. Measurement of Enzymatic Activities

Protein content in root and shoot extract samples was determined according to [9]. Enzymatic activities in root and shoot extracts and in rhizosphere sand were measured spectrophotometrically according to [10]. In plant root and shoot extracts, the activities of laccase, peroxidase, and tyrosinase were measured.

Laccase activity in plant root and shoot extracts was measured with 1 mmol l⁻¹ 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) [11], or with 0.3 mmol l⁻¹ *o*-dianisidine, or with 23 µmol l⁻¹ 2,7-diaminofluorene (2,7-DAF) [1], by using 50 mmol l⁻¹ sodium tartrate buffer at pH 3.5, 50 mmol l⁻¹ sodium acetate buffer at pH 5.0, 50 mmol l⁻¹ Na/K-phosphate buffer at pH 6.0, and 50 mmol l⁻¹ Tris-HCl buffer (pH 7.5). The oxidation rates of the substrates were monitored at λ = 436, 460, and 600 nm, respectively. The activity of peroxidase was measured by monitoring the oxidation rate of the same substrates and at the same pH values in the presence of 0.1 mmol l⁻¹ H₂O₂. The activity of monophenol monooxygenase (tyrosinase) was measured in 4 mmol l⁻¹ 3,4-dihydroxy-DL-phenylalanine and 50 mmol l⁻¹ Na/K-phosphate buffer at pH 6.0 [1]. The oxidation rate of the substrate was monitored at λ = 475 nm.

All the activities are expressed in enzyme units (U), defined as µmol oxidized substrate min⁻¹ and g⁻¹ of root or shoot dry weight or mg⁻¹ protein.

E. Electrophoretic Analyses

Root and shoot extracts, as well as acetone precipitated samples of rhizosphere proteins, were subjected to electrophoretic analysis to determine the enzyme present in the different treatments. Nondenaturing PAGE was used according to [12]. For analysis of enzymes, polyacrylamide gels (12 %) layered with 4 % stacking gels were used.

To reveal peroxidase activity, we stained the gels with 1

mmol l⁻¹ ABTS (pH 3.6), or with 4 mmol l⁻¹ *o*-dianisidine in the presence of 0.1 mmol l⁻¹ H₂O₂.

F. Enzymatic Oxidation of Phenanthrene

The enzymatic oxidation of phenanthrene was carried out by using alfalfa root extracts as a crude enzyme. Phenanthrene (Fluka, Germany) was used as a substrate, dissolved in dimethylformamide (0.2 g l⁻¹), and was added to the reaction suspension to a final concentration of 10 mg l⁻¹. Enzymatic reactions were carried out both with and without a mediating agent. 2,7-DAF (final concentration, 0.02 mmol l⁻¹) was added as a mediating agent to sets of reaction suspensions in order to reveal peroxidase activities. In a total of 2.0 ml, the reaction mixtures contained the following components: 100 µl of root extracts, 50 µl of a solution of phenanthrene, 50 µl of the mediating agent dissolved in acetonitrile, and an additional 100 µl of H₂O₂. The enzymatic reactions were carried out with 50 mmol l⁻¹ Na/K-phosphate buffer at pH 6.0. Controls were run by using the same reaction mixtures and conditions but with boiled enzymes or deionized water instead of active enzyme preparations. The enzymatic reactions were carried out in triplicate in 10-ml tubes closed with sterile plugs in a thermostat at 24°C in the dark. After 24 hours, the reaction was stopped by the addition of 1 ml chloroform, and the substrates were extracted twice for 5 min. The extracts were collected, and the PAH concentration was determined by gas chromatography. The enzymatic oxidation of phenanthrene derivatives was estimated as percentages of substrate elimination in the experimental reaction mixtures, in comparison with the control (inactivated) reaction mixtures. The recovery of all compounds tested was about 90% of their initial concentrations in the samples with heat-inactivated enzymes and in the controls without root extracts.

G. Phenanthrene Analysis

Air-dried sand samples (25 g) were extracted with chloroform (1:1) twice for 5 min, and the extracts were collected and analyzed for phenanthrene residues.

Analysis was carried out by using a Shimadzu 2010 gas-chromatograph, with an Equity-1 (Supelco, USA) nonpolar capillary column, a flame-ionization detector, and He as a carrier and make-up gas. Solution of authentic phenanthrene was used as a standard.

III. RESULTS AND DISCUSSION

Before the enzymatic activity of alfalfa in a PAH-contaminated environment was measured, the growth and development of the plant in the presence of phenanthrene have been evaluated to assess the phytotoxicity of phenanthrene-containing sand.

The toxic effects of PAHs on plants depend both on environmental factors and on pollutant chemical structure, concentration, and exposure [13]-[15]. The phytotoxicity of PAHs was reported to be mainly determined by their lipophilicity, water solubility, and bioavailability [16]. Physical-chemical characteristics of phenanthrene (Mr, 178.2;

water solubility, 1.29 mg l^{-1} at 25°C ; $\log K_{ow}$, 4.57) determine its low uptake by the plant roots and some phytotoxic effects of this pollutant [17]. Plants exhibit responses to toxic compounds only after being exposed to the “threshold concentration” [13], which varies for different compounds and plant species. Baek et al. [14] observed no effect of low (10 mg kg^{-1}) concentrations of naphthalene and phenanthrene, but not pyrene, on the biomass of *Phaserosolus nipponensis* and *Zea mays* grown in soil, whereas a concentration of these PAHs of 100 mg kg^{-1} inhibited plant growth. According to Song et al. [15], the lowest observable concentration that caused adverse effect was 10 mg kg^{-1} for phenanthrene when tested with green onion and 50 mg kg^{-1} for pyrene when tested with wheat.

In our experiment (Table I), the presence of phenanthrene in sand had some stimulating effect on the alfalfa seed germination rate, which was more distinct in the case of a high concentration of pollutant. The germination energy, characterizing the speed of seed germination, decreased in both treatments. The 10-mg kg^{-1} concentration of phenanthrene did not affect plant survival, whereas the 100-mg kg^{-1} concentration reduced this value by 42%.

TABLE I
EFFECT OF PHENANTHRENE ON SEED GERMINATION AND ALFALFA SURVIVAL
IN QUARTZ SAND (% OF CONTROL)

Parameter	Phenanthrene, mg kg^{-1}	
	10	100
Germination rate	2.8%	17.6%
Germination energy	-46.2%	-30.7%
Plant survival	0.5%	-42.0%

Phenanthrene pollution resulted in a decrease in accumulation of alfalfa-shoot biomass, which was inversely proportional to the increase in PAH content (Fig. 1). The accumulation of plant-shoot biomass was reduced by 27 and 63% in the 10 and 100 mg kg^{-1} phenanthrene treatments, respectively. The root system of alfalfa was less affected by phenanthrene pollution: only 100 mg kg^{-1} phenanthrene distinctly reduced root biomass accumulation (by 27%).

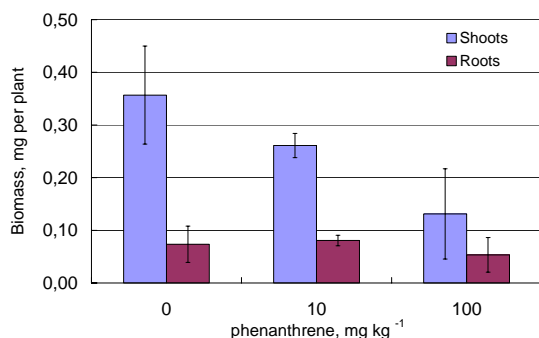


Fig. 1 Effect of phenanthrene on alfalfa-biomass accumulation

Thus, estimation of the growth and development of alfalfa

in phenanthrene-contaminated sand, in comparison with the uncontaminated control, showed that in spite of some stimulation of the seed-germination rate, phenanthrene inhibited the speed of seed germination; lowered shoot-biomass accumulation; and reduced, at the concentration of 100 mg kg^{-1} , root-biomass accumulation and plant survival in phenanthrene-contaminated sand. Consequently, the 100 mg kg^{-1} concentration of phenanthrene had a distinct phytotoxic effect on alfalfa grown in quartz sand. Yet, a clear stress response of the plant to PAH contamination may be expected for alfalfa grown under the influence of phenanthrene.

It is known that plants are capable of enzymatic degradation of organic pollutants in tissues via distinctive plant metabolism [18]. The main plant enzymes involved in transformation/degradation of xenobiotics are phenoloxidases, peroxidases, and cytochrome-P450-monoxygenases [18].

A study of the enzymatic activities of alfalfa shoot and root extracts revealed no significant activity of laccase and tyrosinase. Peroxidases were the dominant enzyme species detected in both root and shoot extracts. Peroxidases are known to be the main oxidative system in the root exudates of different plants [19], [20]. The activity of these enzymes may be stimulated by PAHs [1]. According to Kraus et al. [6], plant-root contact with toxic chemicals like PAHs induces peroxidase activity, which may have an intracellular function as part of a defense mechanism and/or a direct effect on the degradation of aromatics in the external medium.

In our experiments with alfalfa, we estimated peroxidase activity in alfalfa shoot and root extracts by using three test substrates (ABTS, *o*-dianisidine, and 2,7-DAF) at different pH values.

After 8 weeks of cultivation the peroxidase activities in alfalfa shoots ranged from 0.13 to 1.98 U mg^{-1} protein. Among these peroxidases, ABTS peroxidase showed the highest activity, which decreased under the influence of phenanthrene by 40 and 53%, respectively, in the 10 and 100 mg kg^{-1} treatments (Fig. 2a). In contrast, the activity of 2,7-DAF increased from the uncontaminated to the highly contaminated treatment, which was clear when the enzyme activities were calculated per gram of biomass (Fig. 2b). *o*-Dianisidine showed the lowest sensitivity as an alfalfa peroxidase substrate.

Peroxidase activities were considerably higher in the roots than in the shoots of alfalfa and ranged from 2.06 to 46.3 U mg^{-1} protein. In contrast to the shoots, 2,7-DAF was the most sensitive substrate for peroxidase activity of the alfalfa roots (Fig. 3). These findings are in agreement with the data reported by [1] on 2,7-DAF-peroxidases which were the principal oxidative system in the roots and rhizosphere of alfalfa grown in the presence of anthracene and mycorrhiza. According to the data obtained in this study, under the influence of phenanthrene, the activity of 2,7-DAF peroxidase was decreased by 32 and 53%, respectively, for the 10 and 100 mg kg^{-1} treatments (Fig. 3a). At the same time, an increase in ABTS activity was observed. As follows from Fig 3b, the activity of ABTS peroxidase in the 10 mg kg^{-1}

phenanthrene-contaminated sand was 62% higher than that in the uncontaminated control. In the 100-mg kg⁻¹ treatment, ABTS peroxidase was almost threefold more active than in the uncontaminated control sand.

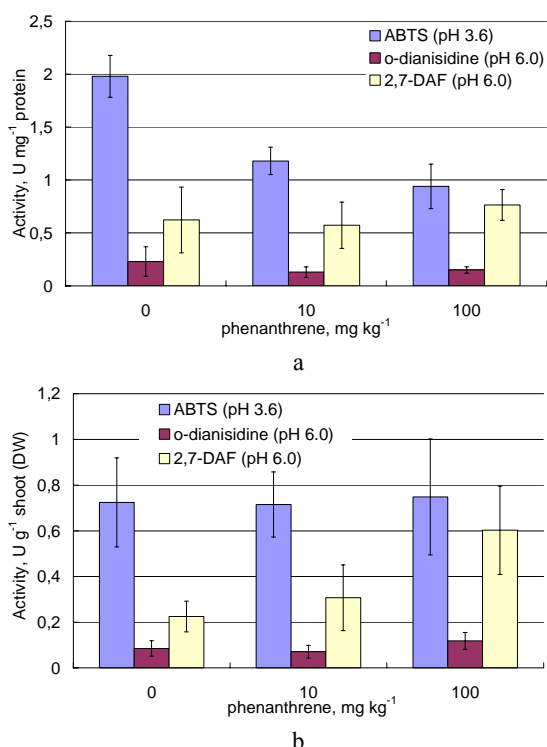


Fig. 2. Peroxidase activities in alfalfa shoot extracts calculated per mg of protein (a) and per gram of biomass (b) in different phenanthrene treatments

Thus, the obtained results revealed qualitative and quantitative differences in the shoot and root peroxidases of alfalfa: ABTS peroxidase was predominant in the shoots and 2,7-DAF peroxidase was predominant in the roots.

Alfalfa shoot and root extracts were subjected to electrophoresis in order to determine peroxidase isoenzymes present in the different treatments.

Nondenaturing PAGE also revealed significant differences in the peroxidase profile of the shoot and root extracts (Fig. 4). Electrophoretic analysis of the shoot peroxidases revealed the presence of six isoenzymes with *R_f* values of 0.04, 0.08, 0.14, 0.2, 0.24, and 0.3. The patterns were similar for all treatments, but for the 10 and 100 mg kg⁻¹ phenanthrene treatments, the bands with *R_f* values of 0.04, 0.08, 0.14, and 0.2 were more intense (Fig. 4a). These differences also point to alterations in the alfalfa-shoot peroxidase profile under the influence of phenanthrene.

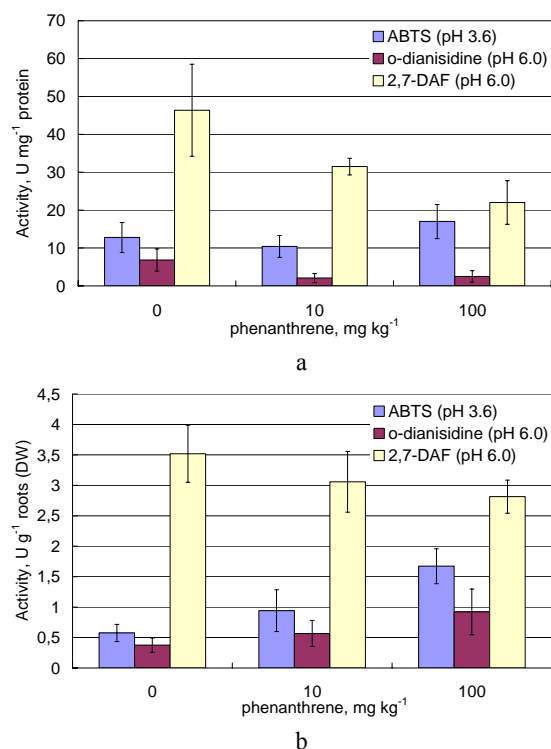


Fig. 3. Peroxidases activities in alfalfa root extracts calculated per mg of protein (a) and per gram of biomass (b) in different phenanthrene treatments

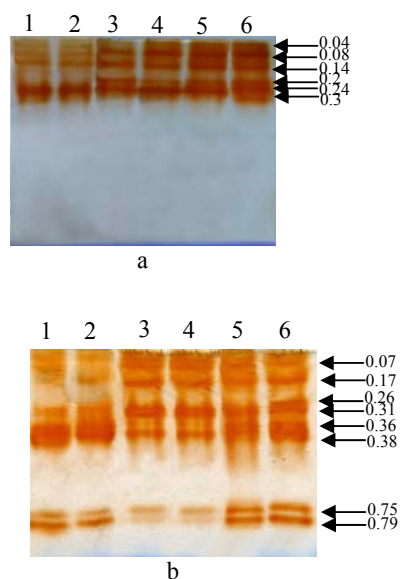


Fig. 4. Electrophoretic patterns (PAGE) of peroxidases from the shoot (a) and root (b) extracts of alfalfa grown at 0 (1, 2), 10 (3, 4), and 100 (5, 6) mg kg⁻¹. Nondenaturing (without SDS or β -mercaptoethanol) polyacrylamide gel was used, and activities were revealed with 4 mM *o*-dianisidine and 0.1 mM H₂O₂.

Electrophoretic analysis of the root peroxidases revealed the presence of eight isoenzymes with *R_f* values of 0.07, 0.17,

0.26, 0.31, 0.36, 0.38, 0.75, and 0.79 (Fig. 4b). The patterns were similar for all treatments.

A study of oxidoreductase activity in uncontaminated and phenanthrene-contaminated rhizospheric sand showed that peroxidase was the principal enzyme. Revealing of peroxidase activity in the planted pots indicated a decisive contribution of the plant to the peroxidase pool in the sand (Fig. 5). In sand with alfalfa, the peroxidase activity was three times higher (0.33 U/g sand) than it was in the unplanted control (0.03 U/g sand). Sand contamination with phenanthrene significantly stimulated peroxidase activity in the alfalfa rhizosphere (by 61 and 48%, respectively, for the 10 and 100 mg kg⁻¹ treatments) but had no distinct effect in the unplanted pots.

Comparison of the electrophoretic patterns from alfalfa root extracts and sand extracts showed that the bands with *R_f* values of 0.17, 0.38, and 0.79 were similar. This points to the alfalfa root origin of active peroxidase in the sand extracts. Thus, an involvement of the alfalfa-root peroxidases in phenanthrene degradation in the rhizosphere sand might be expected in our experiment. Together with the data reported by [5], showing a significant activity of alfalfa-root-excreted peroxidases toward soil humic material, our findings confirm the high peroxidase activity of alfalfa in the rhizosphere environment.

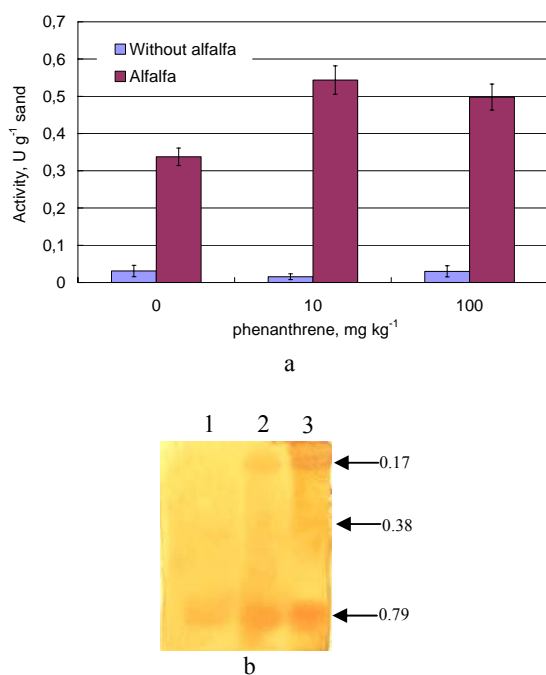


Fig. 4 Effect of alfalfa on the activity of peroxidases (measured with *o*-dianisidine) in sand extracts (a) and electrophoretic patterns (PAGE) of peroxidases from the extracts of sand (b) uncontaminated (1) and contaminated with 10 mg kg⁻¹ (2) and 100 mg kg⁻¹ (3) phenanthrene

Study of the enzymatic activity of alfalfa peroxidase toward phenanthrene showed that the PAH was not oxidized without

a mediator. It is known that the substrate specificity of some enzymes can be broadened with the use of redox mediators [21]. Indeed, the use of a mediating agent (2,7-DAF) in the enzymatic reaction allowed us to observe elimination of 15% of phenanthrene. The use of the mediator and hydrogen peroxide in the PAH-oxidation reaction confirmed the significant peroxidase activity of the alfalfa roots. Oxidation of the PAH in the presence of a synthetic mediator observed in this study does not exclude the existence of such reactions in natural environments, in which the various aromatic substances can mediate similar enzymatic reactions [21].

Estimation of phenanthrene degradation in the sand was unsuccessful in our experiment, because after eight weeks of cultivation, only trace amounts of this PAH were detected in planted and unplanted sand. However, the differences in these amounts were observed (Table 2).

TABLE II
RESIDUAL PHENANTHRENE CONTENT IN THE SAND AFTER EIGHT WEEK
CULTIVATION OF ALFALFA

Treatment	Phenanthrene, mg kg ⁻¹	
	10	100
Without alfalfa	0.093	0.110
Alfalfa	0.038	0.061

In summary, this study has shown that the presence of 10 and 100 mg kg⁻¹ phenanthrene in quartz sand inhibits seed germination energy, plant survival, and biomass accumulation in *Medicago sativa*. The plant stress response to the environmental pollution was an increase in peroxidase activity. Peroxidases were the predominant enzymes in the alfalfa shoots and roots. The peroxidase profile in the shoots differ from that in the roots and had different isoenzyme numbers. ABTS peroxidase was predominant in the shoots, and 2,7-DAF peroxidase was predominant in the roots. Under the influence of phenanthrene, the activity of 2,7-DAF peroxidase increased in the shoots, and the activity of ABTS peroxidase increased in the roots. Alfalfa root peroxidases were the prevalent enzyme systems in the rhizosphere sand. Examination of enzyme activity of alfalfa root peroxidase toward phenanthrene revealed the possibility of involvement of plant enzyme in rhizosphere degradation of the PAH.

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