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Effect of Different Concentrations of Lead Nitrate on Biochemical Parameters of Alfalfa

Reihaneh Sabbaghzadeh (PhD)
Department of Biology, Faculty of sciences, Hakim Sabzevari University, Sabzevar, Iran

Corresponding author: Reihaneh Sabbaghzadeh

Tel: +985144012521

Email: r.sabbaghzadeh@hsu.ac.ir

Address: Department of Biology, Faculty of Sciences, Hakim Sabzevari University, Sabzevar, Iran

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ABSTRACT

Background and objectives: Polyphenols can exert free radical scavenging effects by naturalizing dangerous reactive oxidants. Formation of reactive oxygen species can cause oxidative damage to human cells, leading to various diseases such as cancer, cardiovascular disease, osteoporosis and degenerative diseases. In this study, we investigated effect of treatment with various concentrations of lead (II) nitrate, a toxic and an oxidizing agent, on growth and biochemical parameters of alfalfa (Medicago sativa L).

Methods: Total phenol content was estimated by the Folin-Ciocalteu method. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable free radical was used for assessment of free radical-scavenging activity. Changes in the activity of catalase and peroxidase as well as in the level of proteins, phenol content and malondialdehyde (as marker of lipid peroxidation) were investigated following treatment with different concentrations (0, 8, 12 and 16 mg/l) of lead nitrate for 21 days. All experiments were done in triplicate. Butylated hydroxytoluene and quercetin were used as standard controls.

Results: Treatment with lead significantly altered the level of total phenolic content, proteins, malondialdehyde and the activity of catalase and peroxidase ($P<0.05$).

Conclusion: Our results indicate that lead-contaminated soil can significantly alter biochemical and growth parameters of alfalfa.

Keywords: Lead nitrate, Alkaline phosphatase, Alfalfa.

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INTRODUCTION

Reactive oxygen species (ROS) may be generated in forms of superoxide anion radical ($\cdot O_2-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) during normal metabolic processes or due to exogenous factors (1). Some compounds known as antioxidants are able to delay or inhibit the initiation or propagation of the oxidative chain reaction and thus prevent or repair oxidative damage (2, 3). Recently, antioxidants derived from natural sources have attracted a lot of attention because of their potential application in the food and pharmaceutical industries (4,5). Alfalfa (*Medicago sativa* L.) is an important crop used as livestock feed. One of the most serious pests of alfalfa is pea aphid (Acyrthosiphon pisum) (6).

Polyphenols are known to possess wound healing, antimicrobial and anti-inflammatory properties. Heavy metals disrupt the metabolic processes of living organisms by inducing anatomical changes in primary leaves. A previous study showed that lead nitrate can inhibit plant growth parameters (7-9). Lead (II) nitrate is an inorganic, toxic compound with the chemical formula Pb(NO3)2 that is categorized as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC). Consequently, it must be handled and stored with appropriate safety precautions to prevent inhalation, ingestion and skin contact. Due to its hazardous nature, the limited applications of this compound are under constant scrutiny. All inorganic lead compounds are classified by the IARC as "probably carcinogenic to humans" (Category 2A) (10,11).

Oxidative stress is one of the major mechanisms behind heavy metal toxicity (12). Heavy metals are the most hazardous pollutants as they are non-degradable and can accumulate and cause toxicity in both plants and animals. Among heavy metals, lead is a major contaminant found in soil, sediments, air and water (13). In this study, we investigate antioxidant and growth inhibitory effects of lead nitrate on biochemical parameters of alfalfa.

MATERIAL AND METHODS

The present study was carried out in the Biology Laboratory of Department of Science, Hakim Sabzevari University, Sabzevar, Iran. All chemicals used in the study such as 1, 1-diphenyl-2-picryl hydrazyl (DPPH), quercetin, gallic acid, tert-butyl-4-hydroxy Toluene (BHT), the Folin-Ciocalteu reagent and methanol were all analytical reagents and purchased from Sigma Aldrich and Merk Co.

Plants of *M. sativa* used in the study were transplanted as seedlings (obtained from the Italia Ortosam Co.) into a plot in February 2014. Prior to sowing, the seeds were immersed in sodium hypochlorite (NaOCl) 20% solution of active chlorine for 20 minutes, then in water-alcohol 70% solution for 60 seconds. Later, they were washed with deionized and autoclaved water three times for easy germination. Plants were watered daily. The plants, which initiated vegetative stage in April, were transplanted in laboratory conditions. Plants in the vegetative stage were treated with four concentrations of lead (0, 8, 12 and 16 mg/l) for 21 days with four repetitions per treatment. Then, the plants were collected at various development stages (14-16).

Plant height (end to end) was measured with a tape measure on days 25 and 27 by laying the plants horizontally. Using a top loading balance, fresh weight was recorded. To determine dry weight, the plants were dried at ambient temperature for one week and dry weight was measured in grams.

Total phenol content was estimated by the Folin-Ciocalteu method using gallic acid as the standard (17). The experiment was repeated three times. In addition, BHT and quercetin were used as standard controls (18).

Inhibition rate (antioxidant activity) was calculated using the following formula: Percent inhibition rate = [(AB – AA) / AB] × 100

AB - absorption of blank (t = 0 min)

AA - absorption of test extract solution (t = 30 min).

A variety of methods were used for determining the amount of proteins and enzyme activity. Activity of catalase and peroxidase enzymes, which play important roles in stress relief in the plant, can reflect the amount of stress in the plant. Therefore, an increase in activity of these enzymes indicate higher stress level on the plant. An amount of 0.05 g of the plant samples (without root) was homogenized in a cold porcelain mortar and in an ice pot containing 1

ml of phosphate buffer saline (0.1M, pH 6.8). After centrifugation at 15,000 g, 4 ˚C for 15 minutes, supernatant was separated for measuring activity of catalase and guaiacol peroxidase. Absorbance at 470 nm was recorded every 10 seconds for 2 minutes using a spectrophotometer.

Evaluation of guaiacol peroxidase activity

Activity of the enzyme was measured at 470 nm. This experiment assesses enzymatic conversion of guaiacol to tetraguaiacol in the presence of hydrogen peroxide and the subsequent production of red color. The reaction mixture included phosphate buffer (25 mmol, pH 6.8), guaiacol (20 mmol), ozone (40 mmol) and enzymatic essence (100 µL). The activity of peroxidase was determined through the formation rate of tetraguaiacol per minute with 1 mg of protein.

Evaluation of catalase activity

Activity of catalase was measured at 240 nm. The reaction mixture included phosphate buffer (50 mmol, pH 6.8), deionized water (15 mmol) and 100 µL of enzyme solution. Then, H_2O_2 was added to the medium, which was later mixed with the enzyme solution.

The Bradford protein assay was used to measure the concentration of total proteins (33). Total phenol content was determined by the Folin–Ciocalteu method (19) and lipid peroxidation (20).

Nitrogen content was determined using the Bradford method. The amount of nitrogen was calculated by plotting the standard curve of bovine serum albumin.

The effects of lead on the growth of alfalfa were assessed by measuring concentration of total chlorophyll, chlorophyll a (chla), chlorophyll b (chlb) and carotenoids in the leaves of the growing plant.

Evaluation of lipid peroxidation using malondialdehyde (MDA)

Malondialdehyde is a final product of polyunsaturated fatty acids peroxidation in the cell. An increase in free radicals levels causes MDA overproduction. Therefore, we assessed MDA level as a marker of oxidative stress and antioxidant status.

Data analysis

Samples were observed under a light microscope. Data were analyzed with SPSS (version 19) using one-way ANOVA and at significance level of 0.05.

RESULTS

Environmental conditions are commonly studied as the main constraints to the growth, productivity and distribution of plants. Table 1 shows the physiological characteristics of the plants under different conditions.

Table 1. Physiological characteristics of alfalfa under different conditions

	Organogenesis		Index		
Group	Root Length(mm)	Stems Length(mm)	Number of leaves	Wet Weight(g)	Seed Germination[1]
Control	4.6	2.6	4.22	0.024	63%
8 mg/L	2.14	1.86	4.22	0.031	55%
12mg/L	2.25	1.74	2.94	0.025	53%
16 mg/L	1.91	1.52	3	0.0175	55%

1: two days after culture in agar

The mean level of MDA was 0.010±0.1245 mg/l per gram of fresh weight. Treatment with various concentrations of lead nitrate significantly increased the level of MDA (P=0.0001, F=43.49). This increase was most notable when the plant was treated with 12 and 16 mg/l of lead nitrate (Figure 1a).

The mean concentration of proteins was 0.0478±0.0136 mg/l per gram of fresh weight. Although treatment with lead nitrate increased the concentration of proteins in a dose-dependent manner (F=63.45, P=0.0001), the difference in the concentration of proteins between the groups treated with 12 mg/l and 16 mg/l of lead nitrate was not statistically significant (P=0.31) (Figure 2b).

The mean total phenol content was 0.039±0.0143 mg/l, which differed significantly between the study groups (F=62.67, P=0.0001). The total phenol content of the group treated with 8 mg/l lead nitrate differed significantly from that of the other groups (P=0.01) (Figure 2c).

The mean catalase activity was 0.0573±0.5175. Treatment with different concentrations of lead nitrate had no significant impact on catalase activity of alfalfa (F=3.26, P=0.080) (Figure 2d).

The mean guaiacol peroxidase enzyme activity was 0.0158±0.006. The enzyme activity changed significantly after treatment with different concentrations of lead nitrate (F=16.73, P=0.001). The most significant differences was observed between the group treated with 12 mg/l lead nitrate and the control group (p=0.008) (Figure 2d).

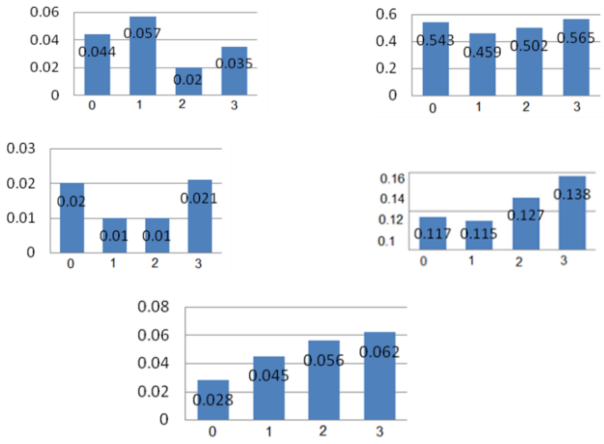

Figure 1. Effect of treatment with different concentrations of lead nitrate on the level of MDA (a), proteins (b), total phenolic content (c) and catalase activity (d) in alfalfa. The Y-axis represent concentration (mg/l per gram fresh weight) or activity, while X-axis represents study groups: control group (0), 8 mg/l lead nitrate (1), 12 mg/l lead nitrate (2), 16 mg/l lead nitrate (3).

DISCUSSION

The growth and metabolism of alfalfa were adversely affected when the plants were exposed to different concentrations of lead nitrate. Lead exerts multiple direct and indirect effects on plant growth, metabolism and some physiological processes. Plant height decreased in a dose-dependent manner following treatment with lead nitrate, which may be due to the decrease in mitotic frequency and lead accumulation in cell wall components, especially pectic substances and hemicelluloses.

Protein content may be affected by heavy metals due to enhanced protein hydrolysis, resulting in decreased concentration of soluble proteins. This may be related to a defect in protein synthesis and or because of the reduction in amino acids essential for protein synthesis.

Based on the results, 8 mg/l and 12 mg/l of lead nitrate did not significantly alter the level of MDA as the marker of lipid peroxidation. However, a relative MDA reduction after exposure to 16 mg/l of lead nitrate was noted.

The activity of catalase did not change significantly, but the activity of guaiacol peroxidase showed a relative reduction. The phenol content of the plant also increased after exposure to 16 mg/ of lead nitrate. However, at this concentration, the protein content of the plant increased remarkably, which could be because of the possible use of nitrate as the Lead (II) nitrate can form coordinate bond with nitrogen and oxygen because of their electron donating capacity (21).

This oxidant functions at high temperatures or in acidic conditions, while lead (II) works best in a neutral aqueous solution (22-24). It has been also reported that lead can retard cell division, differentiation and elongation, thereby affecting the plant growth and development. The decrease in dry weight after treatment with lead nitrate might be due to accumulation of certain nutrients, reduction in photosynthesis and rate of chlorophyll ‘a’ production (25).

Nitrogen and protein content decreased gradually following treatment with lead nitrate. Toxic concentrations of heavy metals may also reduce incorporation of free amino acids into proteins (26, 27).

CONCLUSION

Our results indicate that lead-contaminated soil can significantly alter biochemical and growth parameters of alfalfa.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding publication of this manuscript.

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