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Impact of Cadmium and Lead Heavy Metal Stress on Plant Growth and Physiology of Rocket (*Eruca sativa* L.)

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ABSTRACT

This study was conducted to evaluate the influence of cadmium (Cd) (0, 100, 150 and 200 mg kg^{-1}) and lead (Pb) (0, 1000, 1500 and 2000 mg kg^{-1}) on morphological, physiological and biochemical responses of rocket (*Eruca sativa* L.) in greenhouse conditions. Plant growth, some physiological (membrane permeability, relative water content, stomal conductance, etc.) and biochemical (antioxidant enzyme activity, proline and sucrose content) parameters of rocket plants were altered with Cd and Pb levels. The Cd and Pb content in rocket increased with elevated concentration. Both Cd and Pb stress conditions negatively affected plant growth, photosynthetic activity and chlorophyll content. The negative effect of heavy metals elevated with increased doses of Cd and Pb. The treatment of Cd and Pb significantly elevated antioxidant enzyme activities. Furthermore, the heavy metal stressed plants had more malondialdehyde (MDA), hydrogen peroxide (H_2O_2), proline and sucrose as compared to control plants. This study indicated that rocket plants developed defense mechanisms by regulating enzyme activity and osmolyte accumulation against heavy metal stress.

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Kadmiyum ve Kurşun Ağır Metal Stresinin Rokada (*Eruca sativa* L.) Bitki Büyümesi ve Fizyolojisi Üzerine Etkisi

ÖZET

Bu çalışma, sera koşullarında kadmiyum (Cd) (0, 100, 150 ve 200 mg kg^{-1}) ve kurşunun (Pb) (0, 1000, 1500 ve 2000 mg kg^{-1}) rokada (*Eruca sativa* L.) morfolojik, fizyolojik ve biyokimyasal tepkiler üzerine etkilerini belirlemek amacıyla yapılmıştır. Roka bitkilerinin büyüme, bazı fizyolojik (membran geçirgenliği, nisbi su içeriği, stoma iletkenliği vb.) ve biyokimyasal (antioksidan enzim aktivitesi, prolin ve sukroz içeriği) parametreleri ağır metal stres koşulları altında değişmiştir. Rokanın Cd ve Pb içeriği yüksek konsantrasyonla artmıştır. Hem Cd hem de Pb stres koşulları bitki büyümesini, fotosentetik aktiviteyi ve klorofil içeriğini olumsuz etkilemiştir. Ağır metallerin negatif etkisi artan Cd ve Pb dozları ile daha fazla olmuştur. Cd ve Pb uygulamaları antioksidan enzim aktivitelerini önemli ölçüde artırmıştır. Ayrıca, ağır metal stresli bitkilerde, kontrol bitkilerine kıyasla daha fazla malondialdehit (MDA), hidrojen peroksit (H_2O_2), prolin ve sukroz bulunmuştur. Bu çalışma, roka bitkilerinin enzim aktivitesini ve osmolit birikimini düzenleyerek ağır metal stresine karşı savunma mekanizmaları geliştirdiklerini göstermiştir.

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INTRODUCTION

The ecological pollution emerged by industrialization has caused contamination of soil and water resources. This situation reached dangerous levels in many countries in all the world (Robinson et al., 2001). High heavy metal (Cd^{+2}, Pb^{+2}, Zn^{+2}, etc.) concentrations in agricultural areas were determined to be toxic and the situation is defined as heavy metal stress. Heavy metal accumulation in soils can cause many environmental and human health problems, including microbial activity, soil fertility, biodiversity and yield losses in crops, and even poisoning in foodstuffs. Heavy metals are considered to be one of the most dangerous substances in the environment (Vanlı and Yazgan, 2008). Heavy metals can stay in dangerous densities in ecosystems by moving around food chains. Continuous and usage-related contamination causes a considerable amount of heavy metal content and density in the environment (Okcu et al., 2009).

Heavy metal stress conditions negatively influence plant growth, yield and productivity. These adverse effects occur by affecting the metabolic, physiological and biochemical characteristics in plants. Yield and quality losses are observed in plants grown in industrial areas contaminated with industrial wastewater. It also accelerates aging by reducing photosynthetic activity in the plant. Due to heavy metals, plant roots are usually shortened, thinner or less developed, and in high concentrations cases resulting plants to die (Saklı, 2011).

Heavy metal stress promotes free radical formation, damaging plant tissues and causing oxidative damage in plants. Metal stress leads to producing excessively reactive oxygen species (ROS) that are unstable and harmful to cells and lead to oxidative injury. Excessive amounts of ROS damage nucleic acids, lipids, and proteins. Plants ameliorate different defense mechanisms to prevent from these damages due to heavy metals. These defense mechanisms include plant chelates and antioxidative defense systems with a low molecular weight, thiol-containing and metal-binding polypeptide class. The plants have various antioxidant molecules (ascorbate, glutathione, α-tocopherol) and enzymes (SOD, POD and CAT), which protect themselves against oxidative damage. Antioxidant enzymes have a major role in the defense mechanism developed to heavy metal stress in plants (Unalan, 2010; Ahmad et al., 2017).

The most severe poisonous heavy metals were reported as Cd, Pb and Hg (Okcu et al., 2009). Cd and Pb are dangerous metals, which has a high mobility and negative effects on the plants and all living organisms even at low concentrations (Needleman et al., 1990). Cd can enter the body via the food chain and can lead to health problems (Hassan et al., 2008). Tolerances of plants to heavy metal stress may differ depending on the crop, type of the heavy metal and the duration of exposure to stress. Thus, the type and concentration of heavy metals was reported to be very important on life events in plants (Asri and Sonmez, 2006).

The rocket (*Eruca sativa* L.) is grown mostly in Mediterranean countries and is consumed extensively as a source of nutrition, an aphrodisiac and a medicinal herb. Rocket also is rich in glucosinolates, flavonoids and phenolics, which have antioxidant and anticancer characteristics and are associated with reduced risk of cardiovascular and cognitive disease (Maia et al., 2015). Zhi et al. (2015) stated that rocket plant can be tolerant or moderately tolerant to copper, mercury, chromium, and cadmium, and highly tolerant to lead, nickel and zinc.

There are several studies investigating the influences of Cd and Pb on rocket growth yet, they are limited in details. The present study was conducted to determine the threshold values of rocket in different doses of both Cd and Pb regarding to plant growth, physiology and biochemical characteristics.

MATERIALS and METHODS

The study was conducted in greenhouses of Atatürk University, Plant Production Application and Research Center. Rocket (*Eruca sativa* cv Bengi) was used as plant material.

The Experiment Set up

Seeds were planted in 2-liter pots filled with a ratio of 2: 1: 1 (v: v: v) of soil: sand: manure having around 1.30 g cm^{-3} bulk density. After the seedling emergence, four plants similar in size were left in each pot. The soil moisture in the pots was controlled and irrigated to reach the field capacity as needed. The fertilization was done by using chemical fertilizer to be 250 kg ha^{-1} N, 200 kg ha^{-1} P_2O_5 and 150 kg ha^{-1} K_2O (Esiyok, 2012).

Heavy Metal Applications

In the experiment, 2 pollutants ($PbNO_3$ and $CdSO_4.8H_2O$) ×3 replications x 5 plants were repeated in a total of 120 pots. For heavy metal stress, various levels of Cd (0, 100, 150 and 200 mg kg^{-1}) and Pb (0, 1000, 1500 and 2000 mg kg^{-1}) were mixed into the medium and watered to the field capacity and allowed to incubate for 3 weeks. After this period, rocket seeds were sown. Experiments were completed 50 days after the planting and fresh and dry weights of leaf and root and number of leaf were recorded.

Chlorophyll Content (SPAD)

The chlorophyll content was detected by the SPAD-502 chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan).

Leaf Area

The plant leaf area in each application was measured using a leaf area meter (LICOR, Model: LI-3100, Lincoln, NE, USA).

Membrane Permeability (MP)

A sign of damage caused by plant stress in leaf tissue and especially in cell membranes is the electrical conductivity measurements measured in wet leaf tissues. For this purpose, discs taken from the most recently developed leaves of 2 plants randomly selected from each replicate (1 cm in diameter) were put in glass bottles containing 20 ml of ultrapure water and shaken for 24 hours in the shaker and then the electrical conductivity of the wetting water was determined (EC1). Glass bottles were kept in an autoclave at 121 °C for 20 minutes, after which complete destruction of cells and tissues was achieved, followed by a second measurement (EC2). The membrane permeability values were calculated by calculating the ratio between EC1 / EC2.

Relative Water Content (RWC)

Relative water content (RWC) was determined according to González and González-Vilar (2001).

Photosynthetic Parameters

Photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) were measured on the third fully expanded upper leaves. The measurements were done by using a portable Li-Cor 6400 Photosynthesis System (LI-COR, Lincoln, USA) between 10:00 and 1:00 pm on a sunny day (Ors et al., 2016).

MDA and H_2O_2

MDA and H_2O_2 concentration were determined according to Liu et al. (2014).

Sucrose

The sucrose content was determined by using the method of Wu et al. (2011).

Proline

Proline concentration of the samples was determined at 520 nm by a spectrophotometer (Man et al., 2011).

Cd and Pb Analysis

The leaves of the rocket were dried at 68°C for 48 h and grounded. Cd and Pb content was analyzed by a coupled plasma spectrophotometer (Optima 2100 DV; Perkin-Elmer, Shelton, CT) (Helrich, 1990).

Antioxidant Activity

Assays of antioxidant enzyme activity were performed according to Liu et al. (2014).

Statistical Analysis

The experiment was a randomized plot design with three replications. The data obtained from the experiment were analyzed by using SPSS 18 package program and the means were compared by Duncan multiple comparison test (SPSS Inc., 2010).

RESULTS and DISCUSSION

In our experiment, heavy metal applications had a negative effect on plant growth characteristics in rocket (Table 1). The exposure of the rocket to Cd and Pb stress considerably resulted in reduced growth. The fresh and dry weights of shoots and roots were significantly lowered with the elevation of Cd and Pb concentration. Overall, 200 mg kg^{-1} Cd and 2000 mg kg^{-1} Pb significantly inhibited the growth of the rocket plant. The leaf fresh weight, leaf dry weight, root fresh weight and root dry weight at 200 mg kg^{-1} Cd drastically reduced by 44.7, 51.4, 27.8 and 36.8% as compared to the control, respectively. Similarly, 2000 mg kg^{-1} Pb treated plants had 60.9, 56.8, 40.5 and 63.2% lower in leaf fresh weight, leaf dry weight, root fresh weight and root dry weight, respectively, than the control (Table 1). Root growth inhibition in plants is one of the most important symptoms of Cd and other heavy metals (Kim et al., 2007; Groppa et al., 2008). Many researchers reported earlier that heavy metals have an unfavorable impact on plant growth and have undergone oxidative damage by disrupting metabolism in plants (Schützendübel et al., 2001; Benavides et al., 2005; Gratao et al., 2005). The decline in growth due to cadmium application was explained by cadmium interaction with important metabolic factors such as photosynthesis, transportation of photosynthetic outputs and nutrients in plants (Iqbal et al., 2010).

We also found that Cd and Pb treatments caused reduce on leaf number, leaf area and SPAD values (Table 2). The similar result reported by Opeolu et al. (2010) showing that leaf number and plant height of tomato were adversely affected by Pb applications. In our study, we observed heavy metal treatments caused chlorosis in rocket plants. A decrease in cadmium-induced chlorophyll concentration was shown in barley (Stobart et al., 1985), beans (Padmaja et al., 1990), cucumber (Chugh and Sawhney, 1999), corn and wheat (Zhao, 2011). Similarly, it has been reported that Cd toxicity causes chlorosis, leaf curling and stunting (Moreno et al., 1999; Emamverdian et al., 2015). Heavy metals have been reported to prevent cell division and elongation (Karcz and Kurtyka 2007). Cd stress leads to reduce the concentration of chlorophyll, which is related to the inhibition of chlorophyll biosynthesis (Malik et al., 1992; Vassilev and Yordanov, 1997; Drążkiewicz and Baszyński, 2005). Furthermore, the low chlorophyll concentration due to Cd application

Table 1. Effects of heavy metal applications on some plant growth characteristics in rocket plants

Treatments mg kg^{-1}	Stem diameter (mm)	Leaf fresh weight (g plant^{-1})	Leaf dry weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Root dry weight (g plant^{-1})
Control	3.61 a*	8.56 a	1.11 a	1.92 a	0.19 a
Cd 100	2.85 bc	5.67 b	0.64 bc	1.59 b	0.15 c
Cd 150	2.77 c	4.96 c	0.59 cd	1.53 b	0.13 d
Cd 200	2.48 d	4.73 d	0.54 cd	1.40 c	0.12 e
Pb 1000	2.89 b	5.61 b	0.76 b	1.50 b	0.18 b
Pb 1500	2.81 bc	4.08 e	0.49 d	0.84 d	0.09 f
Pb 2000	2.58 d	3.35 f	0.48 d	0.66 e	0.07 g

*: The means marked with different lower case in same column differ meaningfully ($P < 0.001$)

Table 2. Effect of heavy metal applications on leaf number, SPAD, leaf area, MP and RWC in rocket plants

Treatments mg kg^{-1}	Leaf number no plant^{-1}	Chlorophyll SPAD	Leaf area cm^2 plant^{-1}	MP %	RWC %
Control	9.60 a*	53.07 a	8.10 a	43.99 e	80.17 a
Cd 100	7.74 b	51.20 a	7.13 b	49.39 d	74.40 b
Cd 150	7.58 b	45.87 b	6.23 c	64.45 b	71.30 c
Cd 200	7.23 b	44.87 b	5.11 e	68.56 a	66.72 d
Pb 1000	6.69 b	51.93 a	7.02 b	48.88 d	71.41 c
Pb 1500	6.37 b	50.74 a	6.37 c	58.37 c	65.38 de
Pb 2000	6.76 b	46.23 b	5.62 d	63.55 b	63.09 e

*: The means marked with different lower case in same column differ meaningfully ($P < 0.001$)

was associated with magnesium and iron lack (Greger and Ogren, 1991). Indeed, we observed that Cd caused a decrease in Fe and Mg amount in leaves and roots of rocket plants (data not shown). Marshner (2012) clarified that Cd can affect cell wall extension and cell division, resulting in decreased growth. The most important reason for excessive Cd doses to disrupt the chlorophyll biosynthesis is the inhibition of aminolaevulinic acid synthesis by the protocol of chlorophyll reductase in chlorophyll biosynthesis (Asri and Sonmez, 2011).

The influence of various Cd and Pb levels on the RWC and MP of the rocket plants is shown in Table 2. Cd and Pb applications dramatically increased the MP and decreased the RWC. Metal toxicity may affect membrane permeability, leading to a decrease in water content. It is reported that Cd interacts with the water balance (Costa and Morel, 1994). The decreased RWC observed in our study can be caused by heavy metal-stimulated reductions in hydraulic conductivity (Ehlert et al., 2009). Similarly, earlier researchers showed that heavy metal stress caused a reduction in RWC of several crops (Manousaki and Kalogerakis, 2009; Ahmad et al., 2011). Our findings are in line with Alyemeni et al. (2017) who indicated that Cd elevated the MP in bean. Heavy metals lead to free radicals production and leading oxidative degradation of tilachoid membrane lipids, in such cases it is known that chlorophyll destruction is increased and its synthesis is prevented (Asri and Sonmez, 2011).

As the concentration of Cd and Pb increased, photosynthetic parameters were adversely affected (Table 3). Krupa and Baszynski (1995) reported earlier that heavy metals directly or indirectly affect the photosynthetic process. Cd can also break down the photosynthetic electron chain, causing to the generation of $O_2^{\bullet-}$ and $^1O_2$. Photosynthetic parameters are adversely affected by Cd stress (Asada and Takahashi, 1987; Alloway, 1995). Hasan et al. (2009) determined that Cd had a detrimental impact on photosynthetic activity by causing stomata to close. Although the heavy metals are determined to affect photosynthetic activity, Prasad (1999) reported that Cd is the most important inhibitor of photosynthetic activity. Similarly, Sharma and Dubey (2005) reported that the amount of Pb taken into the plant limits the photosynthesis, disrupts the balance of water and minerals, and adversely affects the hormonal structure and membrane permeability.

The present study showed that Cd and Pb treated plants had more MDA and H_2O_2 content than the control plants (Table 4). Cd and Pb treatments caused to oxidative stress because of enhanced lipid peroxidation and H_2O_2 content. The highest MDA and H_2O_2 were obtained from the 200 mg kg^{-1} Cd treatment. Lipid peroxidation is an indicator of oxidative stress in plants. In this study, lipid peroxidation level increased due to Cd and Pb application. Metal phytotoxicity causes oxidative stress by indirect mechanisms such as increasing the formation of toxic oxygen derivatives and leading to lipid peroxidation (Montillet et al., 2004). Cadmium causes very important damages and oxidative stress in plant metabolism (Gratao et al., 2005).

Table 3. Effects of heavy metal applications on photosynthetic activity in rocket plants

Treatments mg kg^{-1}	**Pn mmol m^{-2} s^{-1}**	**gs mmol m^{-2} s^{-1}**	**Ci µmol mol^{-1}**	**Tr mmol m^{-2} s^{-1}**
Control	9.10 a*	0.88 a	382.78 a	11.04 a
Cd 100	7.76 b	0.35 d	342.78 b	8.73 c
Cd 150	7.34 c	0.32 de	327.18 c	8.10 d
Cd 200	5.63 f	0.30 e	318.00 cd	6.74 e
Pb 1000	7.76 b	0.62 b	348.61 b	10.50 b
Pb 1500	6.90 d	0.52 c	309.08 d	9.10 c
Pb 2000	6.52 e	0.49 c	312.89 d	8.63 c

*: The means marked with different lower case in same column differ meaningfully ($P < 0.001$)

Table 4. Effects of heavy metal applications on MDA, H2O2, proline and sucrose content in rocket plants

Treatments mg kg^{-1}	**MDA nmol g^{-1} dry wt**	**H_2O_2 mmol kg^{-1}**	**Proline µg g^{-1} fresh wt**	**Sucrose %**
Control	4.49 e*	9.90 d	145.64 d	32.82 d
Cd 100	4.88 c	10.59 c	160.33 c	36.54 ab
Cd 150	4.95 bc	11.88 a	163.00 c	37.81 a
Cd 200	5.21 a	11.48 a	182.37 b	35.05 bc
Pb 1000	4.70 d	10.07 d	163.92 c	34.51 c
Pb 1500	4.89 c	11.06 b	218.99 a	38.08 a
Pb 2000	5.05 b	11.03 b	222.21 a	35.97 bc

*: The means marked with different lower case in same column differ meaningfully ($P < 0.001$)

The increase in lipid peroxidation due to Cd application reported earlier in different crops, such as peas (Dixit et al., 2001), cucumber (Zhang et al., 2002) and corn and wheat (Zhao, 2011). Moreover, the onset of lipid peroxidation was seen in bean under cadmium stress (Lozano-Rodriguez et al., 1997).

Proline and sucrose content increased with heavy metal treatments (Table 4). The greatest proline value was observed in 2000 and 1500 mg kg^{-1} dose of Pb treatments while the greatest sucrose values were in 1500 Pb and 150 Cd. Organic osmolytes such as proline and sucrose improve the stress tolerance of plants (Tester and Davenport, 2003).

Cd -tolerant plants have been reported to accumulate compatible osmolytes. As observed in the present study, Irfan et al. (2014) also showed that tolerant cultivars of mustard had greater proline content than sensitive ones in mustard under Cd stress conditions. Zengin and Munzuroglu (2006) found similar observations in sunflower. Moreover, it has been known that the activity of proline synthesizing enzymes could be stimulated under stress environments (Jaleel et al., 2007). Emamverdian et al. (2015) suggested that proline can give a role as protein stabilizer under heavy metal stress.

The influences of the Cd and Pb treatments on SOD, POD and CAT activity in rocket plants are shown in Table 5. Cd and Pb treated plants sustained more SOD, POD and CAT activity than the control (Table 5). Plants activate enzymatic defense systems (SOD, APX, GR, CAT, GPX etc.) under stress conditions. Abiotic stresses conditions enhance ROS in cells that cause the damage of cellular homeostasis. The stimulation of antioxidant enzymes by oxidative stress reflects an overall strategy for dealing with stress (Foyer et al., 1994). SOD, CAT and POD activities have been suggested to lower the lipid peroxidation process in Cd stressed sunflower plants (Gallego et al., 1996). Patel et al. (2016) suggested that the antioxidant enzyme activities, MDA, and proline production increased in heavy metal stressed spearmint (*Mentha spicata*), which was accordance with our results. Similarly, Khavari-Nejad et al. (2013) stated that the phenol, flavonoid, and proline content increased in basil with increasing level of Cd.

Cd and Pb application into the growth medium elevated the concentration of Cd and Pb in both leaves and roots of rocket plants (Table 6). We found that Cd content in the roots was much greater than in leaves. Similar results were found by different authors (Salt et al., 1997; Ozturk et al., 2003; Uraguchi et al., 2009). Heavy metals have been mostly observed to accumulate in the roots. Thus, roots are considered as part of plants that occur toxification (Kabata-Pendias and Pendias, 2001). Cannata et al. (2013) revealed that Cd and Pb content elevated with increased doses of these elements in root media. They also showed that arugula roots had higher metal content than shoots.

CONCLUSION

Both Cd and Pb stress conditions had an adverse effect on plant growth, photosynthetic activity, chlorophyll content, and these effects more drastically occurred when the concentration of two stress factors increased.

Table 5. Effects of heavy metal applications on SOD, POD and CAT activity in rocket plants

Treatments mg kg^{-1}	**SOD EU g leaf $^{-1}$**	**POD EU g leaf $^{-1}$**	**CAT EU g leaf $^{-1}$**
Control	200.33 d *	5715.00 b	145.67 e
Cd 100	216.67 c	6639.67 a	191.00 b
Cd 150	211.67 c	6705.33 a	190.67 b
Cd 200	236.67 a	6683.67 a	227.00 a
Pb 1000	222.67 b	6725.33 a	159.67 d
Pb 1500	216.33 c	6704.67 a	166.00 d
Pb 2000	204.33 d	6635.00 a	177.33 c

*: The means marked with different lower case in same column differ meaningfully (P < 0.001)

Table 6. Effects of heavy metal applications on Cd and Pb content in leaf and root of rocket plants

Treatments mg kg^{-1}	**Leaf Cd**	**Root Cd mg kg^{-1}**	**Leaf Pb**	**Root Pb**
Control	0.32 d*	0.59 d	0.93 d	2.71 d
Cd 100	0.74 c	1.68 c	0.95 d	2.73 d
Cd 150	0.91 b	2.14 b	0.90 d	2.65 d
Cd 200	1.63 a	3.25 a	0.83 e	2.66 d
Pb 1000	0.29 d	0.59 d	1.56 c	4.60 c
Pb 1500	0.28 d	0.59 d	2.16 b	6.90 b
Pb 2000	0.27 d	0.61 d	2.66 a	7.87 a

*: The means marked with different lower case in same column differ meaningfully (P < 0.001)

Cd was more impressive at lower doses than Pb. However, rocket plants developed defense mechanisms by regulating enzyme activity and osmolyte accumulation against the Pb and Cd stress conditions.

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