UDK 620.18:546.48:669.4:633.31
Original scientific article/Izvirni znanstveni članek

ISSN 1580-2949
MTAEC9, 44(5)277(2010)

CADMIUM AND LEAD ACCUMULATION IN ALFALFA (MEDICAGO SATIVA L.) AND THEIR INFLUENCE ON THE NUMBER OF STOMATA

AKUMULACIJA KADMIJA IN SVINCA V RASTLINI LUCERNA (MEDICAGO SATIVA L.) IN NJUN VPLIV NA ŠTEVILO LISTNIH POR

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Prejem rokopisa – received: 2009-12-09; sprejem za objavo – accepted for publication: 2010-06-08

Accurate measurements of the heavy-metal contents in polluted areas are required to assess the potential ecological risks in these areas. Chemical and biological indicators are of interest when they provide information about the concentration and accumulation of heavy metals in the environment. This study was conducted in order to investigate heavy-metal accumulation and the effect on alfalfa (Medicago sativa L.) at different distances from the road. The total content of heavy metals was determined using atomic absorption spectrophotometry. The present results showed a variation that indicated that Pb and Cd had the highest concentration in all the sampling areas near the road. According to the results of the present study, the concentration of heavy metals has an influence on the number of stomata in both the adaxial and abaxial surfaces of the leaves, particularly in locations where the concentrations of Pb and Cd were higher. The results showed that the plant demonstrated a physiological response to the heavy-metal pollution, which suggests that roadside plants are indicators of heavy-metal accumulation and the effect on the environment.

Key words: concentration of Pb and Cd, Medicago sativa L., adaxial stomata, abaxial stomata

Za oceno ekološke obremenjenosti na območjih, ki so onesnažena s težkimi kovinami, moramo izvajati natančne in drage meritve, če hočemo najti kemične in biološke pokazatelje, ki kažejo na kopičenje težkih kovin v okolju – organizmih. Naredili smo raziskavo kopičenja težkih kovin in njihovih posledic na rastlino lucerna (Medicago sativa L.) na različnih oddaljenostih od ceste. Skupna vsebnost težkih kovin je bila določena z atomsko absorpcijsko spektrofotometrijo. Najvišje koncentracije težkih kovin Pb in Cd so bile dosežene v bližini ceste. Prav tako smo ugotovili korelacijo med koncentracijo težkih kovin v rastlini in številom por na obeh straneh listov. Višja koncentracija težkih kovin v rastlini je povezana z večjim številom listnih por. Rezultati so pokazali, da se rastline fiziološko odzovejo na prisotnost težkih kovin, zato je stanje teh rastlin v okolju lahko pokazatelj onesnaženja okolja s težkimi kovinami.

Ključne besede: koncentracija Pb in Cd, Medicago sativa L.,, zgornja pora, spodnja pora

1 INTRODUCTION

The contribution of cars and other road transport to the global emission of atmospheric pollutants is increasing. In parallel with the rapid growth in industrialization, environmental pollution is increasing, with heavy metals like lead and cadmium constituting a significant part. These elements are released into the environment as a result of a wide range of industrial activities. The contamination of heavy metals in the environment is of major concern because of their toxicity and the threat to human life and the environment.[16] Heavy metals are considered to be the main sources of pollution in the environment, since they have a significant effect on its ecological quality. Some heavy metals at low doses are essential micronutrients for plants, but in higher doses they may cause metabolic disorders and growth inhibition for most of the plant species.[12]

In monitoring urban pollution there is a need to consider the materials that cause the occurrence of pollutants. Among toxic metals, lead and cadmium appear to be the most dangerous to the environment.[11] Lead and cadmium cause toxicity and environmental impact, although this toxicity is entirely dependent on the concentration and environmental parameters.

Investigations concerning the concentration of lead and cadmium in alfalfa (Medicago Sativa L.) at different distances from the road and their influence on some morph-anatomic parameters are presented in this study.

2 MATERIAL AND METHODS

Three sites were selected for the collection of 60 plants of Medicago sativa along the Lipjan–Prizren road (**Figure 1**).

1. Location I Lipjan
2. Location II Shtime
3. Location III Suharek

The plant samples were cut off at 2 cm or 3 cm above the soil and stored in plastic bags. In every location the plants were collected at two different distances from the

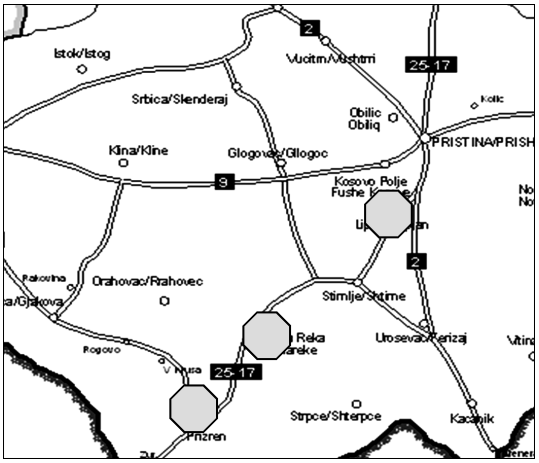

Figure 1: Location sketch map of the investigated area.
Slika 1: Lokacijska skica zemljevida področja raziskav

road where lead and cadmium were determined in the tissue extracts of all the specimens.

All the samples were dried in a thermostat at a temperature of 105 °C for three hours. The digestion of the plant samples was carried out using an acid solution with a 20 : 2 : 1 ratio of nitric acid (HNO_3), perchloric acid ($HClO_4$), and sulfuric acid (H_2SO_4). A portion of 3 g of each sample was weighed and transferred into a 250 mL beaker. The samples were heated to boiling until the white color appeared. Then the samples were acid washed, filtered into the (100 ml) flask, which was added up to the mark with distilled water. The filtered samples were then analyzed for heavy-metal concentration in plant tissues using Atomic Absorption Spectrophotometer.

The stomata number was measured in microscopic fields of leaf impressions taken from the middle parts of leaves. Leaf epidermis imprints of the adaxial and abaxial leaf surfaces were then removed using transparent sticky tape and placed on a microscopic slide. The stomata number was expressed as the number per microscopic field at a magnification of (10X40). Ten fields were assessed in every specimen and the variable was analyzed using Excel computer software.

3 RESULTS AND DISCUSSION

In **Table 1** the concentrations of heavy metals in three different localities are presented. Based on the results related to location 1 (Lipjan), the greatest quantity of cadmium and lead was established in the specimens taken near the road where the average content of cadmium and lead was 1.38 μg/g and 0.814 μg/g, respectively. Research data revealed that recently this highway has been an intensive source of pollution with heavy metals. Heavy-metal accumulation in plant tissues (leaves, stem) was due to the foliar absorption from the surroundings (vehicle sources). According to the results, lead exhibited high levels of contamination closer to the highway. The smallest content of lead (0.21 μg/g) was noted in specimens taken at a distance of 10 m from the highway. The quantity of cadmium in this location was higher in specimens taken near the highway (1.38 μg/g), while the smallest concentration of the cadmium was characteristic for specimens taken at a distance of 10 m from the highway (0.61 μg/g). The general decrease in the concentration of metals with distance from the highway indicates their relation to traffic. This is attributed mainly to the aerial deposition of metal particulates from motor vehicles.[14] The results of the investigation show that the concentration of cadmium in the plant samples varied between 1.38 μg/g and 0.61 μg/g. The accumulation of the selected metals varied greatly among the plant species and the uptake of an element by a plant is primarily dependent on the plant species, its inherent controls and the soil quality.[10]

The alfalfa plant has a high potential to uptake toxic metals like cadmium and lead.[9] Alfalfa has the ability to accumulate concentrations of heavy metals well above the tolerance levels of other plants, which may be due to the presence of specialized chemical functional groups responsible for metal tolerance and accumulation.[1] Various studies have shown the heavy contamination of roadside vegetation with lead. Heavy metals could be absorbed in investigated samples by clay minerals, Fe oxides and/or organic matter. They could also be incorporated into the structures of carbonates.[13]

Based on the results of locality II (Shtime), it is evident that the content of metals grows as the distance between the place of the specimen collection and the road with heavy traffic decreases. The highest content of cadmium and lead in this locality was in specimens taken near the highway (0.29 μg/g, 1.47 μg/g). The concentration of both metals in this location for specimens taken at a distance of 10 m from the highway varied in the range (0.24 μg/g to 1.05 μg/g). These high concentrations may be due to the aerial deposition of heavy metals over a long period of time. A significant correlation was noted between the number of passing petrol vehicles and the lead concentration in the particulate deposits collected from different designated sites (lead in deposits 14.4 μg/g, lead in leaves 1.78 μg/g).[4]

According to the results in location III (Suharek), as distinguished from the first two locations, the highest content of cadmium was in the specimens taken at a distance of 10 m from the highway (2.29 μg/g). The lowest content of cadmium is observed in specimens taken near the highway (1.52 μg/g) **Table 1**. Based on the results obtained, we can conclude that the quantity of lead at this location, as in the two other locations, was greater near the highway (0.79 μg/g), whereas the lowest quantity was observed in specimens taken at a 10m distance from the road (0.16 μg/g) (**Table 2**). Positive and negative correlations are found in the metal concentration at different distances from the highway. Comparatively speaking, almost all the samples collected

Table 1: Cadmium accumulation (µg/g) in alfalfa (Medicago sativa L.) at different distances from the road
Tabela 1: Akumulacija Cd (v µg/g) v rastlini lucerna (Medicago sativa L.) pri različnih oddaljenostih od ceste

Localities and individuals examined	1	2	3	4	5	6	7	8	9	10	X	S	SX	V	Lsd 0.05	Lsd 0.01
P1. 1 m	0.6	0.59	0.28	0.33	0.76	4.52	0.58	2.48	0.47	3.15	1.38	1.47	0.46	1.06	0.16	0.23
P2. 1 m	0.56	0.36	0.29	3.68	0.26	3.05	0.26	3.3	2.69	0.23	1.47	1.49	0.47	1.01	0.36	0.5
P3. 1 m	3.41	0.79	0.26	0.78	0.22	1.14	2.81	0.42	2.94	2.42	1.52	1.23	0.39	0.81	0.21	0.3
P1. 10 m	3.33	0.22	0.39	0.25	0.19	0.26	0.51	0.25	0.35	0.35	0.61	0.9	0.3	1.5	0.16	0.23
P2. 10 m	1.05	0.26	2.83	0.22	0.22	2.69	0.45	0.19	0.51	0.38	2.74	1.1	0.3	1.1	0.36	0.5
P3. 10 m	0.42	0.19	2.87	4.9	2.82	2.74	5.21	0.19	0.29	3.27	2.29	1.9	0.6	0.8	0.21	0.3

Table 2: Lead accumulation (µg/g) in alfalfa (Medicago sativa L.) at different distances from the road
Tabela 2: Akumulacija Pb (v µg/g) v rastlini lucerna (Medicago sativa L.) pri različnih oddaljenostih od ceste

Localities and individuals examined	1	2	3	4	5	6	7	8	9	10	X	S	SX	V	Lsd 0.05	Lsd 0.01
P1. 1 m	1.05	0.26	0.75	0.25	0.51	1.78	0.73	0.23	0.92	1.61	0.81	0.54	0.17	0.67	0.58	0.08
P2. 1 m	0.28	0.29	0.26	0.09	0.72	0.39	0.23	0.03	0.09	0.49	0.29	0.2	0.06	0.7	0.003	0.004
P3. 1 m	0.66	0.82	0.39	0.84	0.61	0.95	1.02	0.58	1.61	0.43	0.79	0.35	0.11	0.44	0.02	0.03
P1. 10 m	0.32	0.32	0.13	0.19	0.23	0.26	0.03	0.12	0.09	0.45	0.21	0.1	0.04	0.58	0.58	0.08
P2. 10 m	0.45	0.19	0.29	0.41	0.16	0.12	0.13	0.16	0.25	0.22	0.24	0.1	0.03	0.47	0.003	0.004
P3. 10 m	0.22	0.09	0.16	0.43	0.09	0.03	0.06	0.13	0.19	0.23	0.16	0.6	0.03	0.68	0.02	0.03

X- Arithmetic mean, S-Standard deviation, SX- Standard error, V-Coefficient of variability, Lsd- Least Significant Difference

Table 3: Number of stomata in the abaxial and adaxial surfaces per 1 mm^2 in alfalfa (Medicago sativa L.) for locality I near the road
Tabela 3: Število por na obeh straneh listov (zgornja in spodnja) na 1 mm^2 pri rastlini lucerna (Medicago sativa L.) v bližini ceste

	Lower leaf				Medium leaf				Upper leaf			
	Upper surface		Lower surface		Upper surface		Lower surface		Upper surface		Lower surface	
No.	X	V	X	V	X	V	X	V	X	V	X	V
1	142.8	0.26	111.9	0.25	170.8	0.23	88.3	0.36	218	0.31	139.9	0.24
2	102.5	0.24	86.9	0.32	44.1	0.39	86.9	0.25	145.8	3	122.2	0.25
3	85.4	0.24	78	0.3	86.9	0.36	153	0.2	54.5	0.35	107.5	0.32
4	98.3	0.37	79.5	0.24	112.2	0.3	91.3	0.37	88.7	0.39	82.4	0.33
5	83.9	0.2	91.3	0.26	88.3	0.23	95.7	0.37	76.5	0.27	129.6	0.29
6	79.6	0.45	98.6	0.44	58.92	0.39	98.6	0.28	84.9	0.27	72.1	0.27
7	67.7	0.2	82.4	0.22	63.3	0.29	57.4	0.18	70.7	0.36	66.2	0.18
8	234.2	0.29	98.6	0.33	64.8	0.3	51.5	0.2	252.2	0.26	67.7	0.38
9	109	0.27	94.2	0.43	91.3	0.27	82.4	0.25	69.2	0.52	107.5	0.54
10	200.1	0.31	69.2	0.4	116.3	0.26	98.6	0.29	141.4	0.23	57.4	0.35
X	120.3		89		114.5		90.3		122.2		95.2	
Lsd 0.05	183.2		129		17.9		80.3		204.9		43.9	
Lsd 0.01	257.1		181.1		25.1		112.8		287.6		61.1	

from site A, which was directly at the border of the road, showed a higher lead concentration than the site-B samples. The average lead concentration in C. sativa in the study area was 3.6 mg kg^{-1}, whereas the average concentration of this metal at the control site was 0.94 mg kg^{-1}.[2]

3.1 Lead and cadmium affect the number of stomata in alfalfa (Medicago sativa L.)

A number of stomata were examined on the abaxial and adaxial leaf surfaces. All the specimens were gathered at the three localities marked on the map. Based on the results (related to the effect of these heavy metals on the number of stomata) it is observed that at location I in three researched leaves (lower, medium, upper) the largest number of stomata per 1 mm2 on the surfaces of the leaves was found in plants examined near the road (156.5 mm^2, 165.7 mm^2, 197.8 mm^2). The difference between the specimens in terms of stomata number on both the abaxial and adaxial surfaces of the leaves was also significant (**Table 3**). In all the specimens examined at different distances from the road the largest number of stomata per 1 mm^2 was on the upper leaf (**Table 4**). In the same location the smallest number of stomata was found in specimens taken at a distance of 10 m from the road (116.4 mm^2, 143.4 mm^2, 159.6 mm^2). It was reported that the lead concentration in leaves of Soybean

Table 4: Number of stomata in abaxial and adaxial surfaces per 1 mm^2 in alfalfa (Medicago sativa L.) for locality I 10 m distance from the road
Tabela 4: Število por na obeh straneh listov (zgornja in spodnja) na 1 mm^2 pri rastlini lucerna (Medicago sativa L.) na oddaljenosti 10 m od ceste

	Lower leaf				Medium leaf				Upper leaf			
	Upper surface		Lower surface		Upper surface		Lower surface		Upper surface		Lower surface	
No.	X	V	X	V	X	V	X	V	X	V	X	V
1	76.5	0.15	82.4	0.42	147.3	0.2	102	0.18	153.1	0.36	79.5	0.42
2	88.3	0.39	107.5	0.35	89.8	0.35	103	0.34	170.8	0.09	103.1	0.27
3	126.6	0.22	98.6	0.29	111.9	0.19	109	0.18	179.7	0.12	131	0.23
4	79.5	0.23	67.7	0.26	178.2	0.27	121	0.39	154.6	0.36	107.5	0.34
5	122.2	0.23	110.4	0.26	181.1	0.14	131	0.31	148.7	0.23	111.9	0.29
6	98.6	0.26	107.5	0.24	162	0.28	119	0.25	188.5	0.17	151.7	0.4
7	172.3	0.27	148.7	0.38	144.3	0.36	118	0.17	107.5	0.32	88.3	0.19
08	86.9	0.28	107.5	0.28	147.3	0.23	121	0.24	100	0.37	75.1	0.35
9	136.9	0.38	73.6	0.24	139.9	0.29	137	0.24	160.5	0.21	119.3	0.28
10	176.7	0.27	178.2	0.14	132.5	0.36	97.4	0.27	232.7	0.37	173.8	0.41
X	116.4		108.2		143.4		115.8		159.6		114.1	
Lsd 0.05	183.2		67.7		213.4		22.3		175.4		15.5	
Lsd 0.01	257.1		95.1		299.5		31.3		146.2		21.8	

X-Arithmetic mean, V-Coefficient of variability, Lsd- Least Significant Difference.

Table 5: Number of stomata in abaxial and adaxial surfaces per 1 mm^2 in alfalfa (Medicago sativa L.) for locality II near the road
Tabela 5: Število por na obeh straneh listov (zgornja in spodnja) na 1 mm^2 pri rastlini lucerna (Medicago sativa L.) v bližini ceste

	Lower leaf				Medium leaf				Upper leaf			
	Upper surface		Lower surface		Upper surface		Lower surface		Upper surface		Lower surface	
No.	X	V	X	V	X	V	X	V	X	V	X	V
1	190	0.26	100.1	0.31	83.9	0.5	60.3	0.17	136	0.5	117.8	0.42
2	219.4	0.16	145.8	0.29	173.8	0.33	191.4	0.23	307.8	0.1	145.8	0.24
3	109	0.36	89.8	0.29	95.7	0.31	60.3	0.4	331.4	0.1	169.3	0.34
4	60.3	0.38	63.3	0.51	122.2	0.18	95.7	0.44	268	0.25	132.5	0.25
5	166.4	0.37	119.3	0.47	114.8	0.28	54.5	0.22	164.9	0.32	120.7	0.47
6	76.5	0.26	45.6	0.31	132.5	0.2	97.2	0.21	85.4	0.43	85.4	0.46
7	82.4	0.25	89.8	0.28	79.5	0.29	89.8	0.28	136.9	0.27	66.2	0.24
8	101.6	0.3	73.6	0.29	175.2	0.18	85.4	0.35	284.2	0.23	114.8	0.45
9	182.6	0.39	104.5	0.3	184.1	0.12	103.1	0.39	142.8	0.27	106	0.34
10	63.3	0.34	54.5	0.28	89.8	0.33	67.7	0.47	94.2	0.32	88.3	0.38
X	125.1		88.6		125.1		90.5		195.1		114.6	
Lsd 0.05	46.5		45.8		27.5		4.6		93.8		35.3	
Lsd 0.01	65.2		64.2		38.6		6.45		131.6		49.5	

X-Arithmetic mean, V-Coefficient of variability, Lsd- Least Significant Difference.

Table 6: Number of stomata in abaxial and adaxial surfaces per 1 mm^2 in alfalfa (Medicago sativa L.) for locality II 10 m distance from the road
Tabela 6: Število por na obeh straneh listov (zgornja in spodnja) na 1 mm^2 pri rastlini lucerna (Medicago sativa L.) na oddaljenosti 10 m od ceste

	Lower leaf				Medium leaf				Upper leaf			
	Upper surface		Lower surface		Upper surface		Lower surface		Upper surface		Lower surface	
No.	X	V	X	V	X	V	X	V	X	V	X	V
1	160.8	0.23	109	0.17	104.5	0.31	73.6	0.33	190	0.27	111.9	0.37
2	73.6	0.24	89.7	0.33	169.3	0.51	45.6	0.42	172.3	0.21	60.3	0.28
3	69.2	0.49	51.5	0.31	150.2	0.4	53	0.35	116.3	0.33	79.5	0.35
4	117.8	0.38	65.4	0.24	97.2	0.4	77.9	0.49	97.2	0.25	67.9	0.22
5	86.9	0.48	60.3	0.4	110.4	0.41	67.7	0.34	182.6	0.35	94.2	0.43
6	113.4	0.21	57.4	0.22	128.1	0.39	54.5	0.27	86.9	0.25	53	0.27
7	72.1	0.31	45.6	0.4	79.5	0.29	50	0.41	119.3	0.6	51.5	0.39
8	67.7	0.21	170.8	0.23	66.2	0.4	57.4	0.42	64.8	0.29	160.5	0.31
9	147.3	0.45	55.9	0.45	88.3	0.36	86.3	0.46	76.5	0.36	53	0.28
10	75.1	0.46	82.4	0.41	151.7	0.3	82.4	0.33	116.3	0.38	60.3	0.33
X	120.3		78.8		89.6		64.8		120.1		79.2	
Lsd 0.05	46.5		45.8		27.5		4.6		93.8		35.3	
Lsd 0.01	65.2		64.2		38.6		6.45		131.6		49.5	

X-Arithmetic mean, V-Coefficient of variability, Lsd- Least Significant Difference.

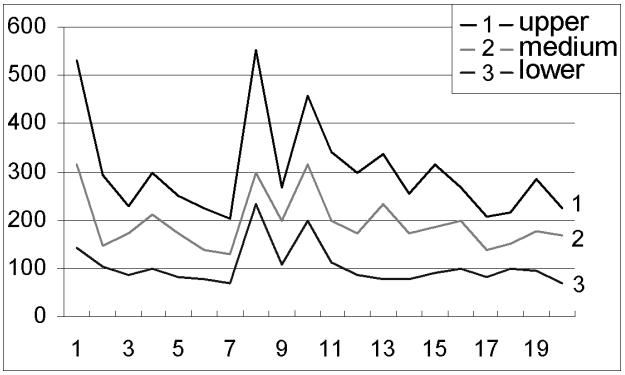

Figure 2: Number of stomata in abaxial and adaxial surfaces of leaves at location III near the road

Slika 2: Število por na obeh straneh listov (zgornja in spodnja) v bližini ceste na 1 mm^2

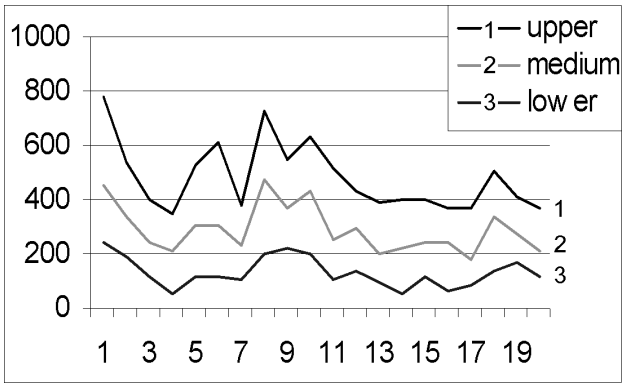

Figure 3: Number of stomata in adaxial and abaxial surfaces of leaves at location III 10 m from the road

Slika 3: Število por na obeh straneh listov (zgornja in spodnja) na oddaljenosti 10 m od ceste na 1 mm^2

(Glycine Max) increased the number of stomata per unit area.[5] Metals can be transported via an apoplastic system and immobilized in the cell walls. Toxic metals become a real threat to plants, mainly when they reach the cytosol of the cell. The data clearly indicate that even very low levels of heavy metals in the soil may have an influence on plant growth.[3]

The number of stomata in the leaf epidermis of Cucumis sativa was increased after cultivation at various Pb rates (10, 20, 40) mg dm^{-3} (1297.6 mm^2 1714.8 mm^2, 1979.5 mm^2). After the treatment with lead the cucumber leaves exhibited a great number of stomata per unit area.[6] Positive and negative correlations were noticed among the metal concentrations and the number of stomata on the upper and lower surfaces of the leaves. Based on the results at location II (**Table 5, 6**) it was observed that both the adaxial and abaxial surfaces of the leaf had a significantly lower stomata number in individuals examined at a distance of 10 m from the road. An increase in the number of stomata per unit area with a simultaneous reduction in the size of the guard cells appeared due to a self-defense system, which is developed in plants under stress conditions and provided the support to the plant for their survival in the contaminated environment.[15]

The stomata number on the leaf for both the adaxial and abaxial leaf surfaces is shown in **Figure 2 and 3**. With the adaxial leaf surface of all individuals examined at locality III the largest number of stomata per 1 mm^2 was found in the upper leaf surface of the specimens gathered from near the road, while the smallest number was in the lower leaf surfaces. A significantly higher stomata number was found on the upper leaf surfaces (95.2 mm^2) compared with two other leaves (medium and lower). The experimental data confirmed the tendency of the increase in the stomata number with industrial pollution at the site II (213.5 mm^2) compared with the control site (205.4 mm^2), and again showed statistically insignificant differences between the control plants and those from the site with high traffic.[8] From this it can be concluded that heavy metals at millimolar concentrations inhibit stomata movements. At higher concentrations, they interfere with metabolic processes and inhibit growth, sometimes leading to plant death.[7]

4 CONCLUSIONS

At three different locations, 60 plant samples of Medicago sativa were collected at different distances from the road and the concentration of Pb, Cd and the number of stomata in the adaxial and abaxial leaf surfaces were determined.

The analysis of the statistical and comparative variance of all examined parameters, the LSD and the coefficient of correlation between the distances from the road were deduced. The greatest content of heavy metals (Pb, Cd) in all localities examined was found in the specimens taken near the road. The lowest quantity of concentration of these metals was found in specimens at a distance of 10 m from the road, with the exception of location III, where the content of Cd was higher at a distance from the road. The concentration of metals decreased with increasing distances from the highway and lead seems to be the most adapted tracer of highway contamination.

The largest number of stomata per 1 mm^2 at locality I was found in the lower leaf (197.8 mm^2). Also at localities II and III, the number of stomata for the medium and lower leaf was lower than for the upper leaf. This study has shown that the street plants are relatively contaminated with heavy metals. Finally, the results obtained in this research work are a significant reference value for future studies of these areas and other regions as well.

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